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DEVELOPMENT OF INNOVATIVE Q-LAMP ASSAY FOR DIRECT DETECTION AND AMPLIFICATION OF INFLUENZA A/B AND RSV GENOMES

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Dottorato in Tecnologie Convergenti per i Sistemi Biomolecolari XXXIII ciclo

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Il lavoro presentato in questa tesi è stato realizzato presso i laboratori di Diagnostica Molecolare DiaSorin sotto la supervisione della Dott.ssa Giulia Minnucci.

Il materiale contenuto nella seguente tesi è strettamente confidenziale. E' stata inoltre presentata richiesta di embargo tesi per un periodo di 3 anni dalla data di conseguimento del titolo.

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ABSTRACT

Despite decades of surveillance and interventions, influenza viruses still represent a public health concern (Stöhr, 2002). Respiratory syncytial virus (RSV) also infects the respiratory system and can cause an influenza-like illness in infants, toddlers and in high-risk adults (E. E. Walsh, 2017).

During epidemics, despite good accuracy of diagnosis based on clinical presentation, specific diagnostic tests are required to confirm virus-specific infection (S. Kumar & Henrickson, 2012).

PCR-based assays are commonly used, requiring prior extraction of viral genomes to be amplified (Merckx et al., 2017). This process is time consuming, needing skilled staff and equipped laboratories.

LAMP (Loop Mediated Isothermal AMPlification) can solve several of these problems, as it is faster, precise, sensitive and specific. This isothermal DNA amplification method relies on the use of a thermostable DNA polymerase with strand displacement activity and six primers specifically designed to recognize eight distinct regions on the target gene. In DiaSorin the technology was improved giving rise to RT Q-LAMP: the coupling of reverse-transcription and amplification activity in one enzyme and the use of fluorescent probes (Q-probes) allows the real time detection and discrimination of multiple RNA targets.

Therefore, the aim of this study is to develop a RT Q-LAMP assay in a multiplex strategy, targeting conserved regions of influenza A, B and RSV viruses to differentially diagnose these infections.

Both reverse-transcription and amplification are performed in a single-step from unprocessed clinical specimens, represented by nasal or

nasopharyngeal swabs (NPS), without viral nucleic acid extraction, allowing clinicians to obtain results in less than 60 minutes.

RT Q-LAMP is performed thanks to Liaison MDX, a small and easy-to use thermocycler, used with its consumable disc named “Direct Amplification Disc” (DAD) which allows the amplification of non-extracted samples, thanks to the combination of centrifugal force and heat treatment.

The so-developed RT Q-LAMP quadruplex assay is able to quickly, sensitively and specifically amplify and discriminate influenza A, B and RSV samples. The thermal protocol has been optimized introducing a sample-processing step ensuring detection of not-extracted viruses. Limit of Detection (LoD) of our assay was defined to be $1x10^3$ copies/mL for all tested viruses and no false positives results were detected on over 100 replicates of Non Template Controls (NTC).

Importantly, it successfully amplified 35 influenza A, 25 influenza B and 11 RSV subtypes and strains assayed with no cross-reactivity with any other microorganisms.

Finally, influenza A, B and RSV RT Q-LAMP assay was validated testing 90 clinical samples from human patients with signs and symptoms of respiratory tract infections and 30 negative clinical samples. RT Q-LAMP results were compared to those obtained with standard diagnostic RT-PCR methods routinely used in the hospital laboratory: clinical specificity was 100% for all the targets assayed and the clinical sensitivity ranged from 87% (for influenza A and RSV) to 90% (for influenza B), the Positive Predictive Value was 100% while the Negative Predictive Value ranged from 96% (for influenza A and RSV) to 97% (for influenza B).

In conclusion, influenza A, B and RSV RT-Q-LAMP assay represents a new tool for the rapid molecular detection and discrimination of influenza A, B or RSV. Its peculiar rapidity, simplicity and reliability makes it perfect for the fast molecular diagnosis of these infections, allowing clinicians to quickly obtain a results and subsequently treat the patient with the most appropriate antiviral medication and to implement infection control measures, especially when the respiratory illness outbreak involves closed settings.

RIASSUNTO

L'influenza è una malattia respiratoria acuta con un considerevole impatto dal punto di vista epidemiologico, clinico ed economico (Stöhr, 2002). L'infezione da RSV causa sintomi simil-influenzali e può portare a gravi complicazioni soprattutto nei bambini e negli adulti ad alto rischio quali anziani, con patologie croniche o immunocompromessi (E. E. Walsh, 2017).

Nonostante la diagnosi basata sui sintomi sia accurata, test diagnostici specifici possono essere fondamentali per confermare l'infezione virale (S. Kumar & Henrickson, 2012). La maggior parte dei test si basano sulla tecnologia RT-PCR (Merckx et al., 2017). Questa metodica richiede una precedente estrazione degli acidi nucleici virali a partire dal campione clinico ma l'estrazione è un processo lungo che può richiedere laboratori attrezzati.

La tecnologia LAMP (*Loop Mediated Isothermal AMPlification*) è veloce e richiede l'uso di strumentazione semplice. LAMP, diversamente da PCR, utilizza una polimerasi con attività *strand displacement* e 6 primers, che riconoscono 8 regioni diverse. Nei laboratori DiaSorin è stata ulteriormente implementata dando origine a RT Q-LAMP: l'utilizzo di un enzima in grado sia di retro-trascrivere che di amplificare il target è stato accoppiato all'utilizzo di sonde fluorescenti (*Q-probes*), la cui riduzione del segnale indica il rilevamento del target. Fluorofori diversi inoltre permettono di discriminare in tempo reale più targets contemporaneamente.

Con il presente progetto di tesi, è stato sviluppato un test RT Q-LAMP in grado di rilevare e discriminare i virus influenzali A, B e RSV. Sia la retro-trascrizione che l'amplificazione vengono eseguite in un unico passaggio senza che l'acido nucleico virale debba essere precedentemente estratto dal

campione clinico. In questo modo si può ottenere un risultato in meno di 60 minuti utilizzando direttamente tamponi nasali o nasofaringei come campioni di partenza. RT Q-LAMP è eseguita grazie ad un termociclatore compatto, sviluppato da DiaSorin e chiamato *Liaison MDX*. Reagenti e campioni vengono caricati su un disco di consumo (*Direct Amplification Disc*_DAD), che permette l'amplificazione di campioni non estratti, grazie alla combinazione dell'effetto termico con la forza centrifuga.

Il saggio finale RT Q-LAMP è in grado di rilevare e discriminare in modo sensibile e specifico i virus influenzali A, B e RSV. Nessuna amplificazione aspecifica è stata rilevata nei 60 minuti di reazione e il livello di sensibilità raggiunta ($1x10^3$ copie/mL di ciascun virus) si è dimostrato adeguato alle richieste cliniche. Inoltre, considerato l'elevato polimorfismo dei virus target, è stato verificato come il test sia in grado di rilevare 35 ceppi di influenza A, 25 ceppi influenza B e 11 sottotipi di RSV. Il saggio inoltre non ha mostrato cross-reattività con altri virus o batteri.

La validazione finale delle performance è avvenuta su 120 campioni clinici (30 negativi e 90 di pazienti positivi a uno dei tre virus di interesse). I risultati ottenuti con il saggio RT Q-LAMP sono stati confrontati con quelli forniti dagli ospedali, ottenuti mediante metodiche classiche di RT-PCR: è stata osservata una specificità clinica del 100% per tutti i virus target, una sensibilità clinica dell'87% (nella rilevazione di influenza A o di RSV) e del 90% (nella rilevazione di influenza B). Inoltre, si sono osservati un valore predittivo negativo del 96% (relativamente alla rilevazione di influenza A o RSV), del 97% (nella rilevazione di influenza B) e un valore predittivo positivo del 100%.

Concludendo, il saggio RT Q-LAMP per la rilevazione e discriminazione di influenza A, B e RSV rappresenta una valida alternativa alle metodiche diagnostiche classiche. È in grado di fornire una risposta estremamente rapida, sensibile e specifica in caso di quesito diagnostico, così che il paziente possa essere trattato velocemente e in modo appropriato e così che possano essere rapidamente implementate misure di controllo e prevenzione nel caso di identificazione di focolai di infezione.

INTRODUCTION

1. INFLUENZA

Influenza viruses belong to the family of *Orthomyxoviridae*. This family consists of five genera (*influenza A virus*, *influenza B virus*, *influenza C virus*, *Thogotovirus* and *Isavirus*), which are classified based on internal nucleoprotein (NP) and matrix (M) proteins (N. Zhang et al., 2020).

The vast majority of the seasonal influenza virus burden is associated with two types of influenza viruses: A and B (type C viruses also circulate in humans but cause less severe disease) (Krammer et al., 2018).

A unique characteristic of influenza A viruses is that they circulate not only in humans but also in domestic animals, pigs, horses and poultry and in wild migratory birds (>100 species of ducks, geese swans, gulls, waders and wild aquatic birds are considered natural reservoirs) (Olsen et al., 2006; Webster et al., 2014).

Influenza A viruses are further classified into subtypes based on the combination of haemagglutinin (HA) and neuraminidase (NA) glycoproteins on their surfaces (Petrova & Russell, 2018). Sixteen haemagglutinin and nine neuraminidase subtypes of influenza A viruses have been isolated from birds (H1 to H16 and N1 to N9) and RNA of additional two haemagglutinin and neuraminidase subtypes has been identified in bats (H17 and H18 and N10 and N11) (Tong et al., 2013). Only three combinations are known to have circulated widely in humans: A/H1N1, A/H2N2 and A/H3N2. Of these, A/H1N1 and A/H3N2 subtype viruses currently cause seasonal influenza virus epidemics (Petrova & Russell, 2018).

A similar animal reservoir does not exist for influenza B viruses but two antigenically distinct lineages of influenza B viruses—Victoria and Yamagata—co-circulate in human beings (Biere et al., 2010; Paules & Subbarao, 2017).

Influenza viruses have a standard nomenclature that includes virus type; species from which it was isolated (if nonhuman); location at which it was isolated; isolate number; isolate year; and, for influenza A viruses only, HA and NA subtype. Thus, A/Panama/2007/1999 (H3N2) was isolate number 2007 of a human influenza A virus taken in Panama in 1999 and it has HA subtype 3 and NA subtype 2 (Bouvier & Palese, 2008).

1.1. Epidemiology

Seasonal epidemics of influenza A and influenza B viruses cause the major burden of disease in humans, with most of the infections occurring in children, although most of the severe cases involve very young or elderly individuals. Seasonal influenza A H1N1 and H3N2 currently circulate in humans, but influenza A H2N2 viruses were the only human circulating influenza A viruses from 1957 to 1968. Before 1918, conclusive evidence of the circulating subtypes is not available. In addition to seasonal epidemics, the introduction of influenza A viruses from either avian or swine populations has led to four pandemics since 1918, with those viruses subsequently becoming seasonal epidemic strains in subsequent years (Figure 1). During pandemics, influenza viruses spread very quickly from the point of origin to the rest of the world in several waves during the year (Figure 1) owing to the

lack of pre-existing immunity, which could also contribute to increased virulence.

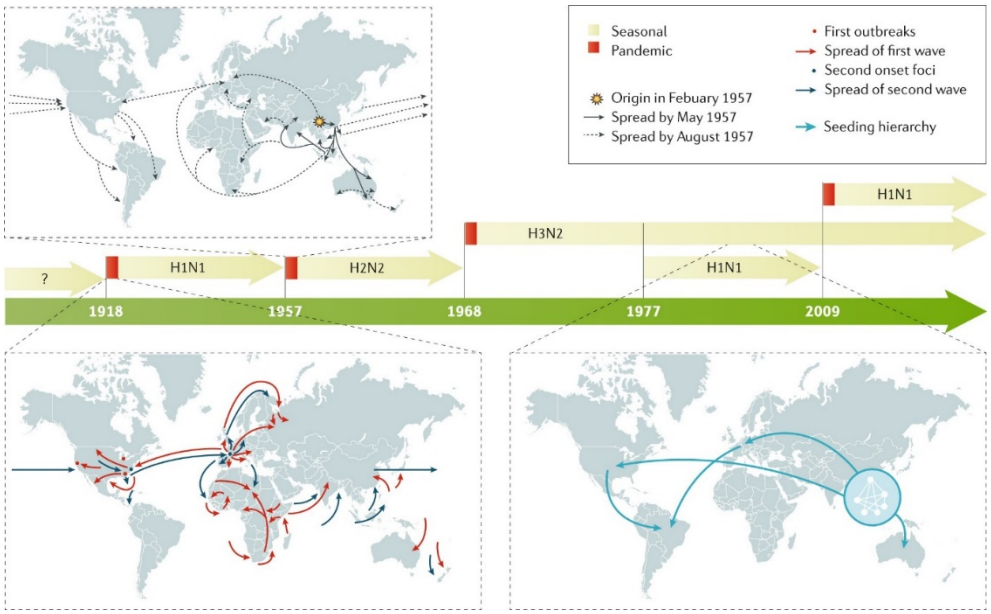


Figure 1. In the past 100 years, four pandemics of human influenza have occurred, with the 1918 pandemic caused by an influenza A H1N1 virus being the most devastating, as it was associated with >40 million deaths (Peter Palese et al., 2006). Influenza A H2N2, H3N2 and H1N1 viruses caused the 1957, 1968 and 2009 pandemics, respectively. In 1977, influenza A H1N1 restarted circulation in humans without causing a pandemic, as the strain was similar to that which preceded the 1957 influenza A H2N2 pandemic. By contrast, the 2009 pandemic influenza A H1N1 virus was antigenically very different from the previous seasonal influenza A H1N1 viruses and replaced them as the circulating influenza A H1N1 strain. Examples of the spreading of human influenza A viruses in the world are shown for pandemic 1918 and 1957 viruses and for seasonal H3N2 viruses (Bahl et al., 2011). For pandemic virus outbreaks, the arrows indicate the first and second waves of transmission. For seasonal influenza A H3N2 spread, the arrows indicate the seeding hierarchy of seasonal influenza A (H3N2) viruses over a 5-year period, starting from a network of major cities in East and Southeast Asia; the hierarchy within the city network is unknown. Seasonal influenza B viruses (not shown) are co-circulating in humans with influenza A viruses (Krammer et al., 2018).

Studies on the transmission of seasonal influenza A virus in humans have proposed that populations in southeast Asia, eastern Asia and/or the tropics act as permanent sources for seeding seasonal epidemics (Rambaut et al., 2008; Russell et al., 2008), whereas other studies indicate that multiple geographical regions might act as seed populations for virus migration (Bahl et al., 2011). However, lack of sufficient sequence data from areas such as Africa, India and South America currently prevents a complete understanding, as viral sequences, which are not routinely obtained during diagnosis, are required to ascertain the relationships between viruses spreading in different areas of the world. Seasonal influenza virus outbreaks typically occur in the winter months, when low humidity and low temperatures favour transmission; two 'influenza seasons' occur per year: one in the Northern Hemisphere and one in the Southern Hemisphere. However, unlike temperate regions, seasonal influenza patterns are very diverse in tropical countries. Climate factors, including minimum temperature, hours of sunshine and maximum rainfall, seem to be the strongest predictors of influenza seasonality (Yu et al., 2013). Seasonal influenza B viruses co-circulate in humans with influenza A viruses and follow the same patterns of transmission.

Pandemic outbreaks are usually associated with the extinction of the previous circulating strains. However, in 1977, influenza A H1N1 viruses, not seen in humans since the 1957 influenza A H2N2 pandemic, started to co-circulate with influenza A H3N2 viruses (Lewis et al., 2011). The 2009 influenza A H1N1 pandemic was caused by an influenza A H1N1 virus that was antigenically very different from the seasonal influenza A H1N1 virus

circulating at the time and resulted in the extinction of the previous influenza A H1N1 human lineage, but it did not result in the extinction of the influenza A H3N2 viruses. Since 2009, influenza A H3N2 and influenza A H1N1 viruses derived from the 2009 pandemic virus and two lineages of influenza B virus are co-circulating in humans (Krammer et al., 2018).

The limited number of pandemic events that have happened makes it very difficult to predict the next pandemic. Human influenza A virus infections with antigenically diverse avian H5N1, avian H7N9, swine H3N2 and other animal influenza viruses are constantly detected in geographical regions where these strains are prevalent owing to the contact of infected poultry or swine with humans. However, no cases of sustained human-to-human transmission have been associated with these viruses, indicating that further adaptations need to take place for these viruses to become transmissible in humans. How feasible it is for these viruses to become adapted to human transmission and retain virulence is unclear. Only a few changes are needed for influenza A H5N1 viruses to become transmissible in ferrets, a host that is often used as an animal model of human influenza virus infection (M. Imai et al., 2012; Richard et al., 2013; Russell et al., 2012).

1.2. Virion structure and organization

By electron microscopy, influenza A and B viruses are virtually indistinguishable. They are spherical or filamentous in shape, with the spherical forms approximately 100 nm diameter and the filamentous forms often in excess of 300 nm length. The influenza A virion is studded with glycoprotein spikes of HA and NA, in a ratio of approximately four to one,

projecting from a host cell-derived lipid membrane (P Palese & Shaw, 2007). A smaller number of matrix (M2) ion channels traverse the lipid envelope, with an M2:HA ratio on the order of one M2 channel per 101 to 102 HA molecules (Zebedee & Lamb, 1988). The envelope and its three integral membrane proteins HA, NA and M2 overlay a matrix of M1 protein, which encloses the virion core. Internal to the M1 matrix are found the nuclear export protein (NEP; also called nonstructural protein 2, NS2) and the ribonucleoprotein (RNP) complex, which consists of the viral RNA segments coated with nucleoprotein (NP) and the heterotrimeric RNA-dependent RNA polymerase, composed of two "polymerase basic" and one "polymerase acidic" subunits (PB1, PB2 and PA) (Figure 2). The organization of the influenza B virion is similar, with four envelope proteins: HA, NA and, instead of M2, NB and BM2.

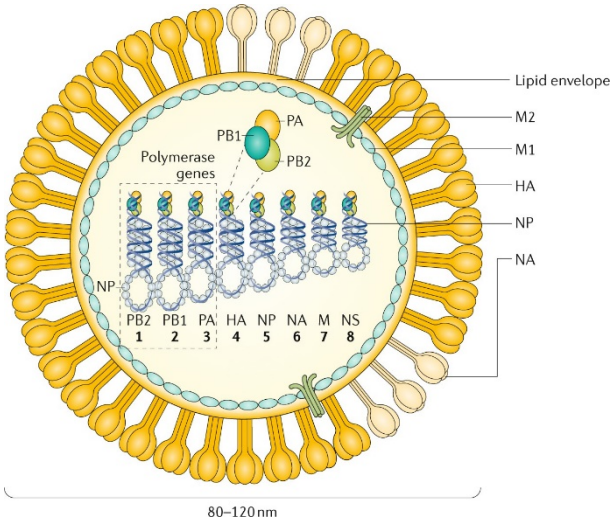


Figure 2. The figure represents an influenza A virus particle or virion. Both influenza A and influenza B viruses are enveloped negative-sense RNA viruses with genomes comprising eight single-stranded RNA segments located inside the virus particle. The RNA genome is bound by the viral nucleoprotein (NP), which is encoded by RNA segment 5. RNA segments 6 and 8 encode more than one protein, namely, the

matrix protein (M1) and membrane protein (M2) — BM2 in the case of influenza B — and the nonstructural protein NS1 (not shown) and nuclear export protein (NEP). The M1 protein is thought to provide a scaffold that helps the structure of the virion and that, together with NEP, regulates the trafficking of the viral RNA segments in the cell; the M2 protein is a proton ion channel that is required for viral entry and exit and that, together with the HA and NA glycoproteins, is located on the surface of the virus anchored in a lipid membrane derived from the infected cell. Finally, the NS1 protein is a virulence factor that inhibits host antiviral responses in infected cells. The influenza viruses can also express additional accessory viral proteins in infected cells, such as PB1–F2 and PA-x (influenza A), that participate in preventing host innate antiviral responses together with the NS1 protein or NB (influenza B), the function of which is unknown. NS1, NEP, PB1–F2 and PA-x are not present in the virus particle or are present in only very small amounts. NB is a unique influenza B virus surface protein anchored in the lipid membrane of the virus particles (Krammer et al., 2018).

1.3. Genome structure

The influenza A and B virus genomes each comprise eight negative-sense, single-stranded viral RNA (vRNA) segments. The eight segments of influenza A and B viruses are numbered in order of decreasing length. In influenza A and B viruses, segments 1, 3, 4 and 5 encode just one protein per segment: the PB2, PA, HA and NP proteins. All influenza viruses encode the polymerase subunit PB1 on segment 2; in some strains of influenza A virus, this segment also codes for the accessory protein PB1-F2, a small, 87-amino acid protein with pro-apoptotic activity, in a +1 alternate reading frame (W. Chen et al., 2001). No analogue to PB1-F2 has been identified in influenza B viruses. Conversely, segment 6 of the influenza A virus encodes only the NA protein, while that of influenza B virus encodes both the NA protein and, in a −1 alternate reading frame, the NB matrix protein, which is an integral membrane protein corresponding to the influenza A virus M2 protein (Hatta & Kawaoka, 2003). Segment 7 of both influenza A and B viruses code for the

M1 matrix protein. In the influenza A genome, the M2 ion channel is also expressed from segment 7 by RNA splicing (R. A. Lamb et al., 1981), while influenza B virus encodes its BM2 membrane protein in a +2 alternate reading frame (Briedis et al., 1982; Horvath et al., 1990). Finally, both influenza A and B viruses possess a single RNA segment, segment 8, from which they express the interferon-antagonist NS1 protein (Dauber et al., 2004; García-Sastre, 2001; Kochs et al., 2007) and, by mRNA splicing, the NEP/NS2 (Briedis & Lamb, 1982; R. A. Lamb et al., 1980), which is involved in viral RNP export from the host cell nucleus.

1.4. Replication cycle

At the cellular level, influenza virus replication (Figure 3) mainly takes place in epithelial cells of the respiratory tract in humans and other mammalians and in epithelial cells of the intestinal tract in birds. The cellular cycle of the virus starts with binding to the target cell. This binding is mediated by the viral HA (which binds to sialic acids present in the oligosaccharides of the glycoproteins at the cellular surface) and is also responsible for the haemagglutination caused by virus particles when incubated with red blood cells. HAs from human influenza viruses bind preferentially to sialic acids linked by an α2,6 linkage to the rest of the oligosaccharide, whereas those from most of the avian influenza viruses favour binding to α2,3-linked sialic acids, as these bonds are the most abundant sialic linkages in the epithelial cells of the human upper respiratory tract and of the avian intestinal tract, respectively (Van Riel et al., 2006; Yamada et al., 2006). After binding, the virus is internalized in an endosome and the endosome is trafficked and

acidified, which triggers a conformational change in the viral HA that induces the fusion of the viral envelope with that of the endosome. As the endosomal pH varies between host species, the pH stability of HA is one of the determinants of viral tropism. Fusion results in the release of virus contents, namely, its genetic material in the form of eight viral ribonucleoproteins (vRNPs), into the cytoplasm. vRNPs are subsequently imported into the nucleus of the infected cells, where transcription and replication of the viral RNA takes place through the enzymatic activities of the viral polymerase complex attached to the vRNPs. Viral RNA replication occurs through a positive-sense intermediate, known as the complementary ribonucleoprotein (cRNP) complex. Viral RNA transcription results in positive-strand mRNAs that are capped and polyadenylated and exported into the cytoplasm for translation into viral proteins. Newly synthesized viral polymerases (PB1, PB2 and PA) and viral NP are imported to the nucleus to further increase the rate of viral RNA synthesis, whereas virus membrane proteins HA, NA and M2 traffic to and get inserted into the plasma membrane. Newly synthesized HA needs to be cleaved into HA1 and HA2 polypeptides by cellular proteases to be functional. The cleavage site of HA is responsible for the tissue tropism of the virus, with all influenza viruses having a cleavage site recognized by extracellular proteases present in respiratory and intestinal epithelial cells, except for the HPAI viruses, which contain a multibasic cleavage site in HA that is recognized by ubiquitous proteases. Viral nonstructural proteins NS1, PB1–F2 (which can be dimeric or multimeric) and PA-x regulate cellular processes to disarm host antiviral responses. Viral M1 and NEP localize to the nucleus at late stages of viral infection, bind to vRNPs and mediate their

export to the cytoplasm, where, through interactions with the recycling endosome, they migrate to the plasma membrane and are bundled into the eight vRNPs. Budding of new virions takes place, resulting in the incorporation of the vRNPs into new virus particles with a membrane derived from the host plasma membrane and containing the viral transmembrane proteins. NA activity prevents non-productive binding of HA of new virions to receptors bearing sialic acid present in the viral glycoproteins and in the membrane of the infected cells, facilitating viral spread. Viral replication results in cell death with pathological implications. In addition, viral products induce a pro-inflammatory response that is responsible for the recruitment of innate and adaptive immune cells, which clear and eliminate the virus, but that in excess induces immunopathology and pneumonia (Krammer et al., 2018).

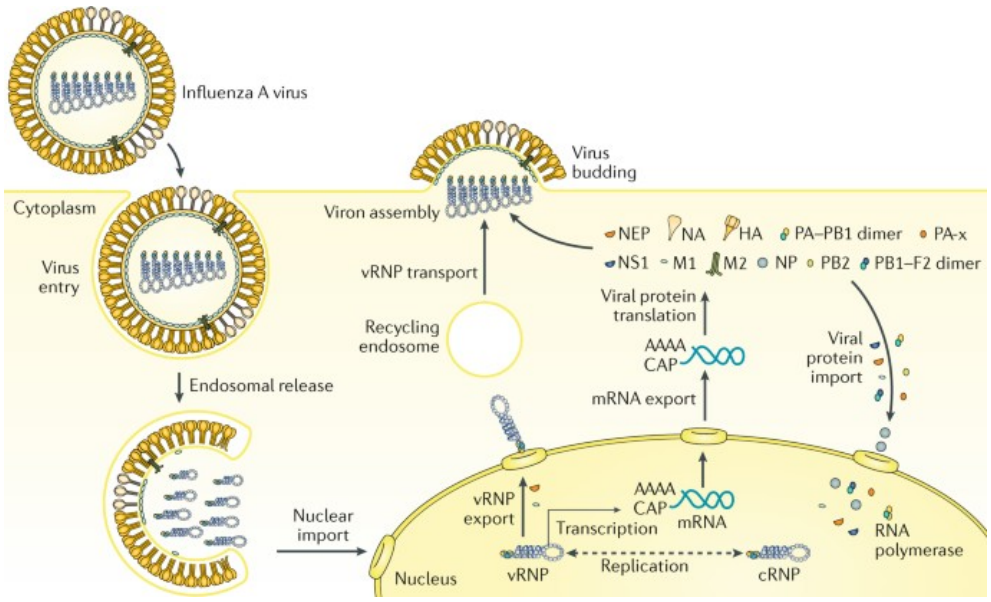


Figure 3. Influenza virus enters the cell by endosomal uptake and release and its negative-sense genetic material in the form of viral ribonucleoproteins (vRNPs) is imported to the nucleus for transcription of mRNA and replication through a positive-sense complementary ribonucleoprotein (cRNP) intermediate. Viral mRNA is translated into viral proteins in the cytoplasm and these are assembled

into new virions together with the newly synthesized vRNPs. PB1–F2 is shown here as a dimer but can also be multimeric. HA, haemagglutinin; M1, matrix protein; M2, membrane protein; NA, neuraminidase; NEP, nuclear export protein; NP, nucleoprotein; NS1, nonstructural protein; PB1, PB2 and PA, viral RNA polymerases (Krammer et al., 2018).

1.5. Antigenic drift and antigenic shift

Two forms of influenza occur globally: epidemic (seasonal or interpandemic) influenza caused by influenza A and B viruses and sporadic pandemics caused by influenza A viruses. These epidemiological forms of influenza result from distinct mechanisms of antigenic variation in the surface glycoproteins of the virus, referred to as antigenic drift and antigenic shift (Paules & Subbarao, 2017). Antigenic drift is a continuous process that occurs in both influenza A and B viruses and results from the accumulation of point mutations in the viral haemagglutinin and neuraminidase genes. Antigenic drift is driven by antibody-mediated selective pressure and a high rate of viral mutations due to the absence of proofreading ability of the viral RNA-dependent RNA polymerase (Bennett et al., 2015; Cox & Subbarao, 2000). Antigenic drift permits the virus to escape immunity induced through previous exposure or vaccination, resulting in seasonal epidemics (Bennett et al., 2015). In temperate regions, influenza epidemics occur annually with a predictable seasonality, whereas in tropical regions they can occur all year round with unpredictable peaks (L. Yang et al., 2015).

In contrast to antigenic drift, antigenic shift refers to drastic changes in the antigenicity of the HA of circulating influenza A viruses; Pandemic influenza A viruses arise as results of antigenic shift; that is, the expression of novel HA and/or NA proteins (Dunning et al., 2020). The HA — and sometimes the NA

— molecules of pandemic viruses are derived from antigenically diverse animal strains of influenza virus, which can be acquired by human influenza strains through reassortment (Figure 4).

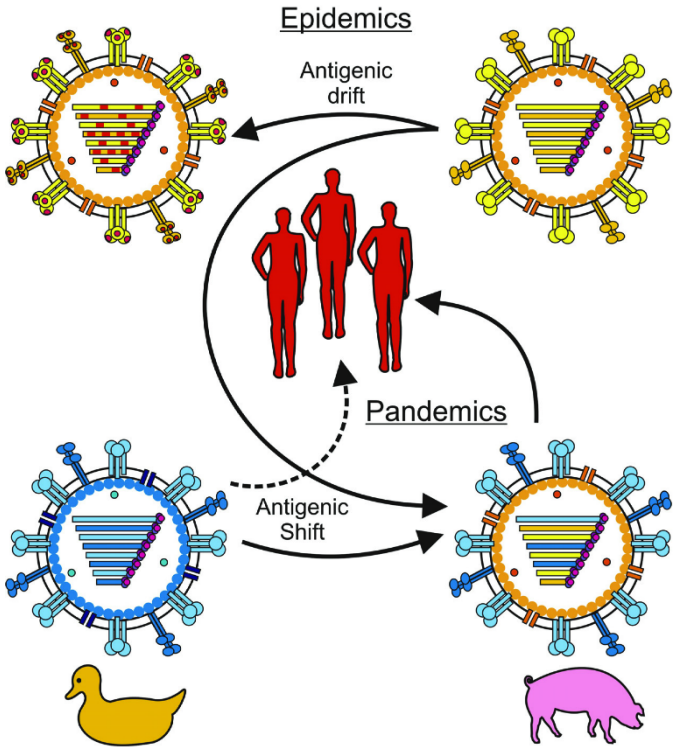


Figure 4. Antigenic drift and shift to escape immunity. The gradual accumulation of mutations, mainly in the highly variable globular head region of HA, causes the influenza virus to escape recognition by virus neutralizing antibodies and allows it to cause seasonal epidemic outbreaks. This phenomenon is called antigenic drift. The introduction of a novel subtype into the human population is called antigenic shift and may cause a pandemic outbreak in the naïve human population when the virus is efficiently transmitted from human to human, since antibodies directed against the novel subtype are absent. Past pandemic outbreaks were caused by exchange (re-assortment) of gene segments between two or more influenza strains, e.g., avian and human. However, recent studies in ferrets suggest that avian influenza viruses, like H5N1, could be directly transmitted from animal reservoirs into the human population, requiring only a small number of adaptive mutations (Richard et al., 2013) as indicated by the dotted line in this figure (Van der Sandt et al., 2012).

2. RESPIRATORY SYNCYTIAL VIRUS (RSV)

Human respiratory syncytial virus (RSV) belongs to the recently defined *Pneumoviridae* family, *Orthopneumovirus* genus. It is a negative sense, single stranded RNA virus that results in epidemics of respiratory infections that typically peak in the winter in temperate climates and during the rainy season in tropical climates. Generally, one of the two genotypes (A and B) predominates in a single season, alternating annually, although regional variation occurs. RSV is a cause of disease and death in children, older people and immunocompromised patients and its clinical effect on adults admitted to hospital is clarified with expanded use of multiplex molecular assays. Among adults, RSV produces a wide range of clinical symptoms including upper respiratory tract infections, severe lower respiratory tract infections and exacerbations of underlying disease, especially in immunocompromised patients or with other comorbidities (Nam & Ison, 2019).

2.1. Epidemiology

In countries with temperate climates, RSV circulates throughout the winter season and peaks between December and January (Rima et al., 2017). In tropical countries, outbreaks of RSV still occur during hot, humid and rainy days in the summer season (Al-Toum et al., 2006).

There are two major RSV groups, A and B, which generally coexist early during an RSV epidemic season, even if temporal and geographic clustering may occur (Hendry et al., 1986; Venter et al., 2001). The antigenic variability between the two groups is determined by variations in the G glycoprotein

(35% homology between G glycoprotein of strains A and B) (T. R. Johnson & Graham, 2004). For this reason, many antibodies targeted to G protein may be subtype specific, while antibodies against the F protein are cross-reactive for RSV A and B. RSV infections with group A are more frequent than those of RSV B and their transmissibility seems to be higher (White et al., 2005). The existence of two groups, A and B and their alternating infection incidences may play a role in the ability of RSV to infect previously exposed individuals and bypass preexisting immune responses (Agoti et al., 2012; Cane et al., 1994). Moreover, additional antigenic variability occurs within the two groups and many genotypes from each group have been described. To date, nucleotide sequence analysis of the G protein has led to the identification of 11 RSV-A (GA1-GA7, NA1, NA2, SAA1 and ON1) (Peret et al., 2000; Venter et al., 2001) and 23 RSV-B genotypes (GB1-GB4, SAB1-SAB4, URU1, URU2, BA1-BA12 and THB) (Auksornkitti et al., 2014; Baek et al., 2012; Dapat et al., 2010; Khor et al., 2013). Different genotypes can co-circulate during an epidemic season and the predominance of one over the other varies by years and location (de-Paris et al., 2014; Pretorius et al., 2013). In particular, the rapid spread of a novel RSV-A genotype (A/ON1, replacing the ancestor A/NA1) has recently been documented in a number of countries (Agoti et al., 2014; Auksornkitti et al., 2014; Tabatabai et al., 2014). The rapid spread of the genotype ON1 may be related to the duplicated sequence within the G gene (Tabatabai et al., 2014; Vandini et al., 2017).

Whether RSV genotype predicts virulence and disease severity remains unclear owing to conflicting data in children (DeVincenzo et al., 2015; Espinosa et al., 2017; Griffiths et al., 2017; Martinello et al., 2002).

RSV is the most frequent cause of bronchiolitis in infants and young children and accounts in the United States alone for approximately 125,000 hospitalizations and 250 infant deaths every year. Global estimates by the World Health Organization indicate that RSV accounts overall for more than 60% of acute respiratory infections in children. Furthermore, RSV is responsible for more than 80% of lower respiratory tract infections (LRTIs) in infants younger than 1 year and annually during the peak of viral season. In summary, RSV is by far the most frequent cause of pediatric bronchiolitis and pneumonia.

Nearly all children are infected at least once by the time they are age 2 years, but peak incidence occurs between ages 2 and 3 months and corresponds to nadir concentrations of protective maternal IgG transferred to the fetus through the placenta. Seasonal outbreaks occur each year throughout the world, although onset, peak and duration vary from one year to the next. In the United States, the annual epidemics usually begin in November, peak in January or February and end in May.

However, the epidemiology of RSV differs widely across latitudes and meteorological conditions. For example, at sites with persistently warm temperatures and high humidity, RSV activity tends to be continuous throughout the year, peaking in summer and early autumn. In temperate climates, RSV activity is maximal during winter and correlates with lower temperatures. In areas where temperatures remain colder throughout the year, RSV activity again becomes nearly continuous. Thus, RSV activity in communities is affected by both ambient temperature and absolute humidity,

perhaps reflecting meteorological combinations that allow greater stability of RSV in aerosols.

Morbidity and mortality of RSV disease are higher in premature infants and in infants with chronic lung disease (e.g. bronchopulmonary dysplasia, cystic fibrosis and interstitial lung diseases) or hemodynamically significant congenital heart disease. Development of bronchopulmonary dysplasia or other chronic respiratory conditions amplifies the risk of severe infections by limiting pulmonary functional reserve, distorting airway architecture and promoting a proinflammatory milieu (Piedimonte & Perez, 2014).

Most studies of the epidemiology of RSV in adults have important limitations as they focus on selected groups at risk and older studies used non-molecular techniques with decreased sensitivity for the diagnosis of RSV. Data derived from clinical testing are limited by reduced frequency of testing in the ambulatory setting, particularly among older people and adults who present later to clinical care (Binder et al., 2017; Chartrand et al., 2015).

Despite most initial and severe infections occurring during early childhood, RSV is increasingly recognized as a common cause of respiratory illness in adults. RSV is the causative agent in up to 12% of medically attended acute respiratory illnesses (Colosia et al., 2017). Although less than 1% of adults affected are estimated to need admission to hospital (Hall, 2001), RSV is the third most commonly identified viral cause of admission, accounting for approximately 177 000 hospital admissions and 17 000 excess deaths, mostly in adults over the age of 65 (Falsey et al., 2005; Jain et al., 2015; Thompson et al., 2003). The average length of stay for patients admitted to hospital with RSV is three to six days, with an overall mortality of 6-8% (Colosia et al.,

2017). Among adults admitted to hospital who have a positive RSV test, approximately 10-31% need to be admitted to an intensive care unit, with 3-17% needing mechanical ventilation (Colosia et al., 2017).

One large prospective study conducted over three successive influenza seasons (2006-09) found RSV to be associated with a higher rate of hospital admission than both human metapneumovirus and influenza (15.01, 9.82 and 11.82 per 10 000 residents, respectively) (Widmer et al., 2012). Among all adults, RSV attributed mortality is generally estimated to be less than 1% (Falsey et al., 2014; Widmer et al., 2014), although RSV may account for as much as 25% of the excess winter mortality that historically was attributed solely to influenza (Thompson et al., 2003).

Previous infection with RSV does not convey persistent immunity even in the presence of significant antibody titers, although higher titers may attenuate the course of the disease. Consequently, subsequent infection is common, can recur within the same viral season and occurs across all age groups. The first episodes of infection typically occur in the first 2 years after birth and tend to be the most severe because of the limited immunologic protection discussed above, smaller airway size and unique structural and functional features of the developing respiratory tract (e.g., lack of interalveolar pores and channels and different innervation patterns) (Piedimonte & Perez, 2014).

Most subsequent infections remain confined to the upper respiratory tract and run a milder course, although the illness may still progress to an LRTI, especially in elderly and immunodeficient patients, usually characterized by more severe symptoms (Piedimonte & Perez, 2014).

As most RSV infections in adults are not their first infection, most patients experience mild to moderate clinical disease. Risk factors for progression to viral pneumonia and complications include Down's syndrome, compromised immunity (including patients receiving chemotherapy or chronic immunosuppression for connective tissue disease/vasculitis), underlying lung disease (especially asthma) or heart disease, old age, frailty, living in a long term care facility and living at high altitude (Beckhaus & Castro-Rodriguez, 2018; Chatzis et al., 2018; Choudhuri, 2006; Ivey et al., 2018). Among immunosuppressed patients, the greatest burden is seen in recipients of hematopoietic stem cell transplants (HSCT) and lung transplants, who have an incidence of RSV of 12-16% (Milstone, 2006; Weinberg et al., 2010). Although the rates of medically attended RSV vary depending on season and diagnostic methods, outcomes are generally consistent in showing increased incidence with age, with the highest annual mortality rate from RSV associated pneumonia in adults aged 65 years or above (7.2 per 100 000 person years) (Colosia et al., 2017; Nam & Ison, 2019; Thompson et al., 2003).

2.2. Virion structure and organization

RSV was reclassified in 2016 into the family *Pneumoviridae,* genus *Orthopneumoviridae.*

Before this reclassification the taxon *Pneumoviridae* was a subfamily within the *Paramyxoviridae* (Griffiths et al., 2017). RSV is a medium sized (120-300 nm diameter) pleomorphic enveloped virus with a non-segmented, negative sense, single stranded RNA genome (~15-16 kb), which encodes 11 proteins: two non-structural and nine structural proteins (Figure 5).

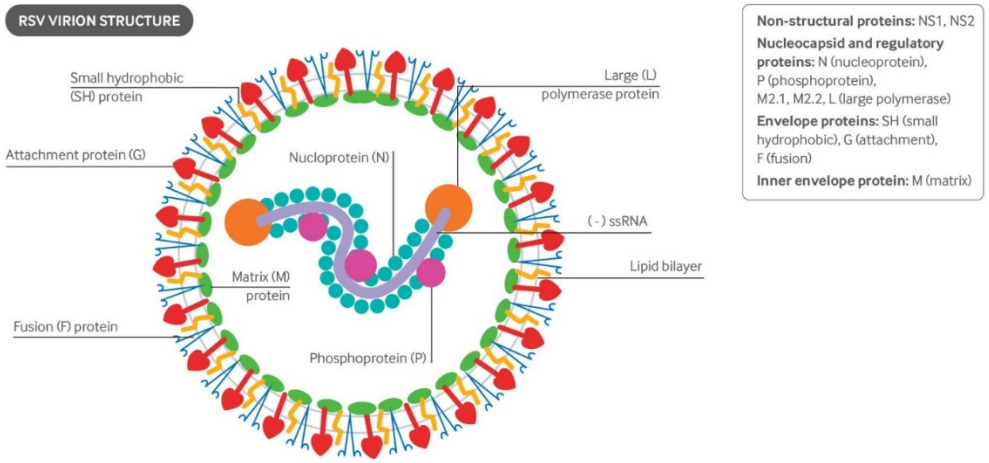


Figure 5. The structure of respiratory syncytial virus: viral envelope of RSV contains three transmembrane glycoproteins: attachment glycoprotein (G), fusion protein (F) and small hydrophobic protein (SH). Matrix proteins (M) are present on the inner side of the viral envelope. Viral RNA is tightly encapsidated by nucleoproteins (N) and the large proteins (L), phosphoproteins (P) and M2-1 proteins that mediate viral RNA transcription. M2-2 protein regulates viral RNA synthesis (Nam & Ison, 2019).

G (attachment), F (fusion) and SH (proposed viroporin) are hydrophobic transmembrane surface glycoproteins that are important for infectivity, as maximally efficient fusion requires participation of all three of these surface glycoproteins (Gan et al., 2008; E. E. Walsh & Hall, 2015). These surface glycoproteins can also serve as the focus of protective antibodies and are therefore potential therapeutic targets. G primarily mediates virus attachment to host cells by targeting ciliated cells of the airways. Following attachment, F undergoes structural changes into a post-triggered form that allows viral penetration by fusing viral and host cellular membranes, as well as facilitating micropinocytosis (Colman & Lawrence, 2003; Krzyzaniak et al., 2013). This further promotes the fusion of infected cells to adjacent uninfected cells, resulting in the characteristic RSV syncytia from which the

name of the virus derives. Both F and G protein are antigens that produce protective immunity and are important in the initial phases of infection as targets for antibody-mediated neutralization. G has moderate to high sequence diversity and defines the antigenic groups A and B, whereas F is highly conserved between strains and is recognized by broadly cross neutralizing antibodies; it is therefore an attractive candidate as an RSV vaccine antigen (McLellan et al., 2013; Vekemans et al., 2019). The pre-fusion form of the F protein is more effective than the post-fusion form in stimulating higher titers of optimally neutralizing antibodies (Cullen et al., 2017; Krzyzaniak et al., 2013).

2.3. Genome structure

The RNA genome is packaged into the viral particle as a non-segmented negative-sense molecule and codes for key internal structural proteins (Figure 6) (matrix protein [M] and nucleoprotein [N]), proteins required for a functional polymerase complex (phosphoprotein [P] and polymerase [L]), nonstructural proteins involved in evasion of the innate immune response (NS-1 and NS-2), externally exposed transmembrane glycoproteins (small hydrophobic protein [SH], glycoprotein [G] and fusion protein [F]) and the regulatory M2 proteins (M2-1 antitermination protein and M2-2, involved in transcription/replication regulation) (P Palese & Shaw, 2007).

The RSV genome contains noncoding regions in the 3’ and 5’ termini, called the leader and trailer regions, respectively. Within the 3’ leader sequence, the individual nucleotides important for both replication and transcription have

been finely mapped using mutagenesis (Fearns et al., 2002; McGivern et al., 2005).

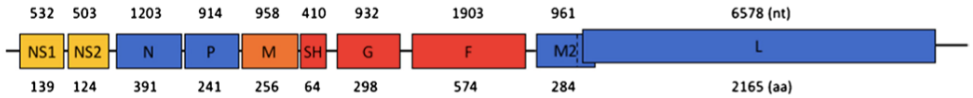


Figure 6. RSV Genomes and proteins: a map of negative-sense RNA genome where "nt" and "aa" indicate nucleotides and amino acid lengths, respectively (Taleb et al., 2018).

There is a great deal of similarity in the nucleotides needed for the polymerase to perform either replication or transcription (Fearns et al., 2002; McGivern et al., 2005); the leader sequence itself can be split into 3 regions going from 3' to 5': polymerase initiation site (nucleotides 1 to 15), the RSV-N encapsidation and elongation signal (nucleotides 16 to 34) and transcription signal (nucleotides 36 to 43) (Griffiths et al., 2017; McGivern et al., 2005).

2.4. Replication Cycle

RSV attachment and entry are mediated by the G and F glycoproteins, with no apparent contribution by SH. Entry occurs by fusion of the viral envelope with the cell plasma membrane (Figure 7). There is also evidence of entry by clathrin-mediated endocytosis, although endosomal acidification was not required and thus this pathway involves the same fusion mechanism as at the plasma membrane (Kolokoltsov et al., 2007; Srinivasakumar et al., 1991). Genome transcription and replication occur in the cytoplasm and the virus can grow in enucleated cells and in the presence of actinomycin D, indicating a lack of essential nuclear involvement.

By extrapolation from prototype members of *Mononegavirales*, incoming nucleocapsids engage in transcription by preformed polymerases (primary transcription). The availability of newly synthesized soluble viral N and P proteins promotes elongation of RNA replication products, leading to the production of full-length encapsidated antigenomes and genomes. Progeny genomes engage in transcription (secondary transcription) and RNA replication. Transcription and RNA replication occur concurrently. RSV mRNAs and proteins can be detected intracellularly at 4–6h after infection and reach a peak accumulation by 15–20h.

At this time, transcription may be downregulated in favor of RNA replication and the production of genomes needed for packaging and this appears to be mediated by the M2-2 protein (Bermingham & Collins, 1999). However, there is no evidence of a change in the relative molar amounts of the various viral mRNAs during the infection time course (Fearns et al., 2000). The release of progeny virus begins by 10–12h post infection, reaches a peak after 24h and continues until the cells deteriorate by 30–48h.

As already noted, RSV-infected cells develop large cytoplasmic inclusion bodies that become evident by 12h post-infection (Lifland et al., 2012; Lindquist et al., 2011). These have been shown to contain the viral N, P, M2-1 and L proteins, as well as viral RNA. As noted, the inclusion bodies are thought to be sites of RNA synthesis. More recently, the viral inclusion bodies have been shown to sequester key cellular signaling components and thereby inhibit cellular responses to infection. These include Mda5 and MAVS involved in interferon induction (Lifland et al., 2012) as well as p38 mitogen-activated protein kinase and O-linked N-acetylglucosamine tranferase

involved in stress responses and stress granule formation (Fricke et al., 2013). Infected cells develop filamentous surface projections that bear viral glycoproteins and may give rise to viral filaments. Infected cell lines develop syncytia that are a major viral cytopathic effect and lead to the destruction of the monolayer, but that are much less evident in differentiated, polarized epithelium *in vitro* and *in vivo* (J. E. Johnson et al., 2007; L. Zhang et al., 2002).

RSV assembly and budding occur at the plasma membrane. In polarized cells, this occurs at the apical surface (Roberts et al., 1995; L. Zhang et al., 2002). These regions contain localized virus-modified lipid rafts involving all three viral surface proteins and the M protein (Henderson et al., 2002; Jeffree et al., 2003; McCurdy & Graham, 2003; McDonald et al., 2004; Yeo et al., 2009). The minimum viral protein requirements for the formation of virus-like particles capable of delivering the viral genome to target cells are the F, M, N and P proteins (Teng & Collins, 1998) and expression of these proteins induced the formation of viral filaments (Utley et al., 2008). Both genome and antigenome have been detected in virions, suggesting a lack of selective packaging. Genome-containing nucleocapsids are much more abundant in the infected cell and are correspondingly more abundant in virions. RSV appears to hijack cellular apical recyclin endosomes for budding, a pathway that is distinct from that described for a number of other enveloped RNA viruses (Brock et al., 2003; Collins et al., 2013; Utley et al., 2008).

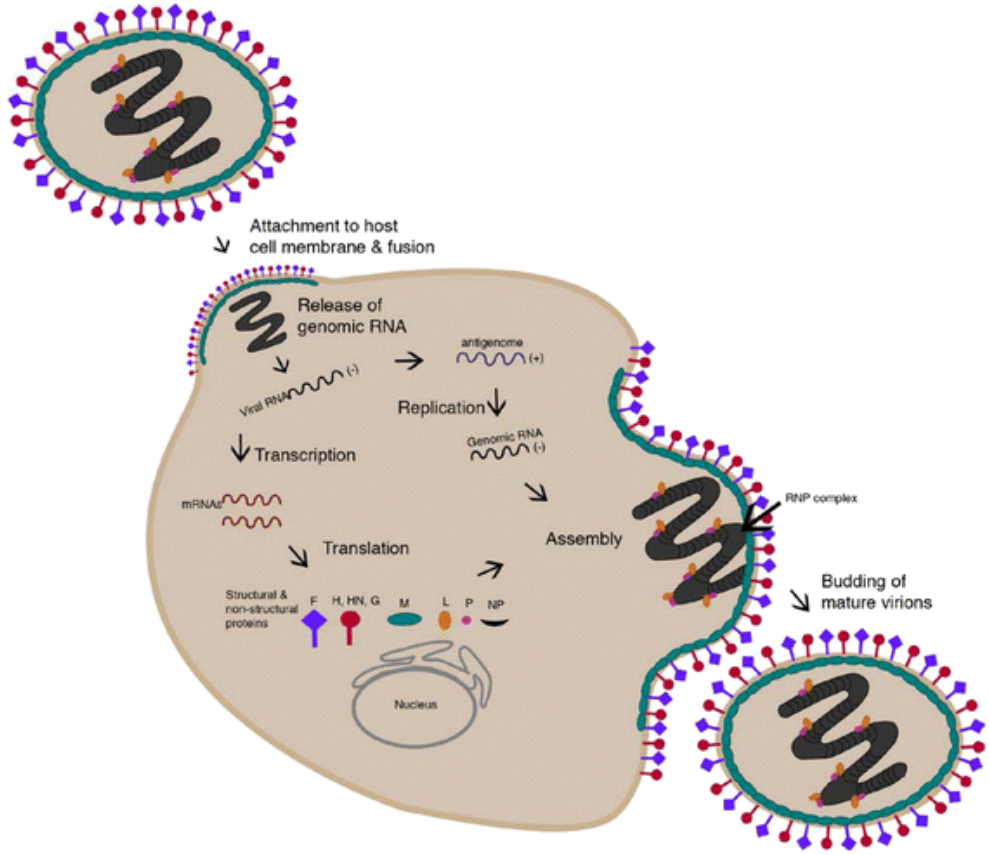


Figure 7. The life cycle of RSV (Shahriari et al., 2016): infectious cycle of RSV begins upon attachment of the virion to the apical surface of polarized, ciliated airway epithelial cells (L. Zhang et al., 2002). The viral attachment (G) glycoprotein associates with cell surface factors and facilitates the initial attachment step (S. Levine, 1977; S. Levine et al., 1987). After fusion, the helical ribonucleoprotein complex (RNP) is released into the host cell cytoplasm. Transcription and replication occur in the cytoplasm in viral inclusion bodies that serve to concentrate viral products (García-Barreno et al., 1996; García et al., 1993; Rincheval et al., 2017). The viral RNA-dependent RNA polymerase (RdRp) complex is responsible for transcribing viral mRNA and synthesizing positive-sense anti-genome intermediates needed for replication of new negative-sense genomes for packaging into virions (Noton & Fearns, 2015). In addition to performing non-proofreading polymerase functions, the RdRp caps and polyadenylates viral mRNAs. Assembly of RSV virions occurs at or near the plasma membrane (Gower et al., 2005; Ke et al., 2018). After budding from the apical membrane of polarized epithelial cells, virions detach and are released in a M-dependent maturation process as filamentous particles ~130 nm in diameter and 0.5–12 micrometres in

length (Förster et al., 2015; Ke et al., 2018; Roberts et al., 1995). Over time, the M layer dissociates from the viral membrane, creating non-filamentous regions in the virion that ultimately lead to spherical or pleiomorphic particles that are thought to be less infectious, likely owing to a premature conversion of the F protein from the prefusion to postfusion conformation (Killikelly et al., 2016; Liljeroos et al., 2013).

3. DIAGNOSIS OF INFLUENZA AND RSV

Accurate and fast diagnosis of the causative viral pathogens is important to select the appropriate treatment, save people's lives, stop the epidemics and reduce unnecessary use of antibiotics. A number of diagnostic approaches, including virus isolation, as well as some emerging molecular-based approaches, have been used to detect influenza and RSV viruses in clinical laboratories (N. Zhang et al., 2020).

3.1. Non-molecular approaches

3.1.1.Viral culture

Viral culture is the gold standard for diagnosing influenza viral infections (N. Zhang et al., 2020). This approach includes inoculation of the corresponding cell lines, such as Madin Darby canine kidney (MDCK), A549 and rhesus monkey kidney (LLC MK2), with clinical samples, propagation for 7 to 10 days to monitor the development of cytopathic effect (CPE) and final confirmation of influenza virus infection by hemadsorption using erythrocytes, specific antibody staining or immunofluorescence microscopy (Vemula et al., 2016).

Speaking about RSV diagnosis, a variety of cell lines have been used in clinical laboratories, including human embryonic kidney and HEp-2 (Wright et al., 2005) tube cultures and shell vials (Smith et al., 1991), A549 conventional and shell vial cultures (Matthey et al., 1992) and rhesus monkey kidney (RhMK) and human foreskin fibroblast conventional cultures and shell vials (Pedneault et al., 1994; Rabalais et al., 1992).

Commercially available products such as R-mix shell vials, a combination of mink lung cells (Mv1Lu) and human adenocarcinoma A549 cells, have been used to support and allow for the detection of RSV when paired with an antibody detection method (Barenfanger et al., 2001; Bell et al., 2014). Occasionally but not routinely, RSV propagation has been described in H358 (Valdovinos & Gómez, 2003) and human adenocarcinoma MRC-5 (Levitz et al., 2012) cell culture tubes.

A more recent advancement in the last decade has been the use of co-cultured cells, where two or more cell lines that support the growth of a variety of respiratory viruses are mixed and grown as monolayers on shell vials (Ginocchio, 2007). A mix of specific monoclonal antibodies is used for viral detection at 24 h, 48 h, or 5 days post-inoculation. The advantage of this method over conventional shell vial culture is the ability to simultaneously culture several viruses in the same shell vial without requiring different cell culture setups for individual viruses. Several co-cultured cell line systems are currently available that support the growth of multiple respiratory viruses, including influenza A and B viruses, parainfluenza virus types 1 to 3, adenovirus and respiratory syncytial virus (RSV). The R-Mix shell vial system (Quidel/Diagnostic Hybrids Inc. [DHI], Athens, OH) combines a human adeno-

carcinoma cell line (A549) and mink lung epithelial cells (Mv1Lu). The R-Mix Too system combines the Madin-Darby ca-nine kidney (MDCK) and A549 cell lines, which are excellent for isolation of respiratory viruses and have the advantage of not supporting the growth of the highly pathogenic severe acute respiratory syndrome coronavirus (SARS-CoV).

Although viral culture is playing less of a role in routine clinical diagnosis of influenza by laboratories performing molecular assays, shell vial-based techniques with hybrid cell lines were utilized widely by a large number of laboratories that did not have access to molecular assays during the pandemic (Hayden et al., 2010).

Even in laboratories using molecular methods for routine clinical diagnostics, isolation of virus in culture is highly valuable in some clinical situations, such as for (i) further characterization of viral strains from patients with unusual or severe disease; (ii) sequencing of strains with mutations (e.g., shifted melt profiles) in molecular assays; (iii) culture confirmation of suspected false-positive PCR results; (iv) culture confirmation of positive PCR results for uncommon infection sites, such as the myocardium, brain, or other specimen types that molecular assays are not typically validated for; and (v) differentiating prolonged shedding of viral nucleic acid from true viral replication (S. Kumar & Henrickson, 2012).

In the last years, there has been a notable decrease in the use of culture-based detection techniques in U.S. hospital-associated laboratories, as the use of molecular tests has increased, especially from the period 2007 to 2011 (Rabon-Stith et al., 2013). Until nonbiased detection systems become more widespread and reliable, culture-and antigen detection-based assays play an

important backup role especially in cases where single nucleotide polymorphisms lead to false-negative PCR results (Hawkinson et al., 2013).

Other roles for culture may include propagation of virus for further genetic characterization, a trend that may decrease with whole-genome sequencing and analysis of antiviral compounds (Kwanten et al., 2013; Lundin et al., 2013) and virucidal agents (Griffiths et al., 2017; Thevenin et al., 2012).

In the last years, viral culture has increasingly been replaced by molecular assays as the modality of choice for influenza diagnosis in most clinical laboratories. Disadvantages of culture include delayed availability of results (3 to 14 days) and a significantly lower sensitivity, particularly for samples with low viral loads. Moreover, culture approaches lack sensitivity, often quite significantly, compared to nucleic acid amplification assays for the diagnosis of both influenza and RSV infections (Cho et al., 2014). This was also notable when nucleic acid amplification test methods were compared to culture for lower respiratory tract specimens (Griffiths et al., 2017; Kadmon et al., 2013).

3.1.2.Immunoassays

Antibodies to influenza virus appear after ~2 weeks and peak 4 to 7 weeks after infection. A variety of serological tests, including hemagglutination inhibition (HAI) assays, complement fixation assays and enzyme immunoassays (EIAs), exist (Katz et al., 2011; Marie Louise Landry, 2011; Petric et al., 2006).

Currently, several FDA-approved enzyme-linked immunosorbent (ELISA) - based tests are available for diagnosis of influenza virus infections. However,

ELISA-based tests often show lower sensitivity compared with nucleic acid-based methods (P. Zhang et al., 2014).

A ∼4-fold change or increase in influenza virus antibody titers in paired acute-and convalescent-phase samples obtained at least 2 weeks apart establishes the serologic diagnosis of influenza. The requirement for 2 samples obtained weeks apart renders this method not useful for clinical diagnostic testing, but it can be helpful for making the diagnosis retrospectively. Although not useful as a diagnostic test for most clinical scenarios, serology can play an important role in some situations. These include the following: (i) in the absence of previous vaccination or infection with a particular strain, such as when a novel virus emerges to which there is no preexisting cross-reactive immunity, a single specimen with a positive titer is diagnostic (Katz et al., 2011); and (ii) serology can help to establish a diagnosis of novel or seasonal influenza virus infections beyond the period when culture and/or PCR tests would be positive. Thus, in patients with a history of influenza-like illness (ILI) but who have stopped shedding the virus or in patients with asymptomatic infections, serology may be the only available option to make a diagnosis.

Serology is a valuable tool for conducting seroepidemiological studies, which are essential for estimating the true burden of infection with an antigenically novel strain. Many such studies performed during the 2009 pandemic not only helped to ascertain the geographic extent and epidemiologic spectrum of the novel virus after its emergence but also elucidated other important aspects of the virus, such as preexisting cross-reactive immunity in older adults, which were relevant for public health

recommendations regarding vaccine prioritization (Hancock et al., 2009; Petric et al., 2006).

Serologic studies were also critical for evaluating the immunogenicity of the 2009 H1N1 influenza A vaccines, which was important for formulating recommendations on dosing for different age groups. Serologic assays have been and will continue to be used for antigenic characterization of circulating influenza viruses to determine antigenic drifts or shifts and formulate vaccine recommendations during both pandemics and annual epidemics.

Thus, serologic investigations are an important component of the pandemic response and maintenance of serologic testing capabilities in select laboratories will be one critical component of future pandemic preparedness activities (S. Kumar & Henrickson, 2012).

ELISA and immunofluorescence tests are traditionally also used to identify RSV. However, a modified ELISA method has been developed, targeting RSV F protein and it can detect RSV within 25 minutes at low cost (Rochelet et al., 2012) (Table 1). The immunofluorescence assay can rapidly detect RSV antigens using a fluorescence-tagged primary or secondary antibody. For example, the direct fluorescent antibody assay (DFA), which requires a certain number of cells in the specimen, with a sensitivity and specificity of 94% and 96.8%, respectively, is widely used for detection of RSV in clinical laboratories because of its simplicity and rapidity. For this reason, this assay has particular use in resource-limited countries since it can potentially eliminate prolonged hospitalization and unnecessary use of antibiotics (Bhandary, 2016; N. Zhang et al., 2020). In details, DFA testing requires a swab that allows for an appropriate number of epithelial cells to be collected and is largely applicable

to appropriately collected nasopharyngeal specimens. Specimens that lack enough cells or originate from other sites in the respiratory tract are not appropriate for this type of testing. This is suggestive that historic surveillance data that relied heavily on DFA testing may have been underestimating the true impact of RSV-A and RSV-B on influenza-like illness rates in pediatric and adult populations and rates will change as surveillance systems shift between different detection approaches (Rabon-Stith et al., 2013). However, depending on workflow and resources within the laboratory, DFA testing as an adjunct to molecular test methods may provide an option for RSV testing in high-risk patients such as hematopoietic stem cell transplant patients (Moreira et al., 2013). Prior to the broader utilization of easier-to-use molecular diagnostic assays, DFA testing historically provided a more rapid response than lab-developed and batched molecular assays for RSV (Rath et al., 2012). The sensitivity of DFA testing can be excellent in pediatric patient populations. However, the sensitivity of DFA testing is significantly decreased in adult patient populations, especially compared to commercial nucleic acid amplification tests (Table 1) (M. L. Landry & Ferguson, 2014).

Test type	Assay	Study test population age (yr)	% sensitivity	% specificity	Comparison method
First-generation POC	Binax Now	All ages, all specimens (0 to >89)	70.8	97.8	Composite standard: cell culture and DFA
	Binax Now	<22	72.0	97.6	Composite standard: cell culture and DFA
	Binax Now	<5	72.4	97.6	Composite standard: cell culture and DFA
	Binax Now	Pediatric	90	100	Composite standard: cell culture and/or PCR
	Binax Now	Not defined	81.7	98.7	Composite reference
	BD Directigen EZ RSV	Pediatric	90	94	Composite standard: cell culture and/or PCR
	BD Directigen EZ RSV	Pediatric	79.8	89.5	Laboratory-developed RT-PCR
Second-generation POC	3M rapid detection RSV	All ages, all specimens (0 to >89)	86.3	95.8	Composite standard: cell culture and DFA
	3M rapid detection RSV	<22	87.2	95.6	Composite standard: cell culture and DFA
	3M rapid detection RSV	<5	87.9	95.8	Composite standard: cell culture and DFA
	BD Veritor system	<6	81.6	99.1	Prodesse ProFlu+ RT-PCR
	BD Veritor system	<6	79.1	96.8	Laboratory-developed RT-PCR
	RSV K-SeT antigen test	<6	79.1	95.8	Laboratory-developed RT-PCR
	Sofia	<18	87.7	94.7	Traditional cell culture
DFA on primary specimen	SimulFluor	0–17	93.5	99.6	Composite reference
	Cytospin- DFA	Mostly adult hospitalized patients	73.9	99.8	Laboratory-developed RT-PCR
	Bartels; Trinity Biotech	Pediatric	94.1	96.8	Laboratory-developed RT-PCR
Culture	R-Mix Too followed by IF	Not defined	63.2	ND	Composite reference
	R-Mix followed by IF	0–17	86.5	100	Composite reference
	WI38, RMK, and D^3 Ultra DFA respiratory virus screening and ID kit	Not defined	56.9	100.0	Composite reference
Rapid molecular test	Cepheid Xpert Flu/RSV	Not defined	97.9	100	Laboratory-developed RT-PCR
	Cepheid Xpert Flu/RSV	Not defined	90.6	99.4	Laboratory-developed RT-PCR
Multiplex molecular	AdvanSure	Not defined	96.8	100	Composite reference
	Seeplex RV15 ACE	Not defined	94.7	100	Composite reference
	ResPlex II Panel v2.0	0–17	84.0	100	Composite reference
	Seeplex RV15	0–17	100	97.7	Composite reference
	xTAG RVP	0–17	88.2	100	Composite reference
	xTAG RVP Fast	0–17	91.7	100	Composite reference
	xTAG RVP Fast	0–84	94.7	99.2	Laboratory-developed real-time RT-PCR
	Simplexa FluA/B and RSV	Mostly adult hospitalized patients	91.3	98.9	Laboratory-developed RT-PCR

[a]Abbreviations: ND, not defined in study; IF, immunofluorescent staining; ID, identification.

Table 1. Diagnostic methods for detection of RSV in patient populations (adapted from (Griffiths et al., 2017)

Other groups have identified that in pediatric populations, compared to nucleic acid amplification testing, DFA test sensitivities are probably highest in the first 3 days of infection (Shafik et al., 2011).

Semiconductor quantum dots can be used for biological and biomedical applications because of their unique size-dependent optical and electronic features. The assay detects RSV F protein using thioglycolate (TGA)-coated cadmium telluride (CdTe) particles, which are bioconjugated with RSV anti-F protein mAb (Tripp et al., 2007). It overcomes some of DFA's disadvantage, such as relatively low sensitivity, because of the background staining and the rapid fading of the dye. Also, this assay is more sensitive than RT-PCT. By probing F and G proteins with QDs, confocal microscopy could detect the progression of RSV infection in the HEp-2 cell line and this method was found to be more sensitive compared to RT-PCR (Bentzen et al., 2005).

Lateral flow immunoassay (LFIA) is another rapid RSV detection method based on an immunochromatographic technique using the samples of nasal washes or aspirates. Many LFIA kits are now available in the market, such as BD Directigen EZ RSV, Binax Now RSV, RSV Respi-Strip, Remel Xpect and QuickLab RSV Test (Borek et al., 2006; Selvarangan et al., 2008; Slinger et al., 2004). The sensitivity and specificity of the above mentioned kits are normally higher than 90% and 95%, but they differ by manufacturer (N. Zhang et al., 2020).

The above-described conventional diagnostic methods generally have lower sensitivity and specificity relative to molecular methods (Table 1). With

the development of laboratory approaches for virus diagnosis, isolation of viral pathogens from secretion samples has become much easier than before (N. Zhang et al., 2020).

3.2. Molecular-based approaches

Molecular assays have increasingly been accepted as the gold standard diagnostic method for detection of influenza virus. Although several amplification methods have been described, the majority of current assays, particularly those utilized in clinical laboratories, are based on the PCR amplification format. Advantages of PCR assays over more conventional viral culture-based diagnostics for influenza include significantly higher sensitivities and short turnaround times. Additional valuable features of PCR-based assays include (i) the ability to test for several targets concurrently and thereby provide type and subtype information, detect other respiratory viruses with overlapping seasonality and detect influenza virus coinfections; (ii) the ability to be implemented using automated and high-throughput platforms that have the potential for testing large sample numbers and requiring minimum technician time; and (iii) the ability to be adapted rapidly for detection of novel targets. These features were key to the critical role that molecular assays played during the influenza pandemic of 2009 (S. Kumar & Henrickson, 2012).

Nucleic Acid Amplification Tests (NAAT) assays based on polymerase chain reaction (PCR) detect virus-specific genetic materials, rather than viral antigens or antibodies. Therefore, optimal extraction of viral genetic materials is required. One of the advantages of NAAT over RIDT is that NAAT

is able to identify different subtypes of influenza viruses. Trombetta's group found that NAAT shows much higher sensitivity for both influenza A and influenza B viruses, despite its relative lower specificity (Trombetta et al., 2018). A variety of NAAT assays, such as reverse transcriptase-PCR, loop-mediated isothermal amplification-based assay (LAMP), DNA-microarray-based and sequencing-based tests, are appearing for diagnosis of influenza viral infections in humans (Vemula et al., 2016).

3.2.1.Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RT-PCR is the most traditional yet powerful NAAT approach for identification of influenza and RSV viruses in most diagnostic labs around the world. Considered a gold standard assay for influenza diagnosis, RT-PCR involves three essential steps: (1) extraction of viral RNA from clinical specimens; (2) Reverse transcription of viral RNA to a single-stranded cDNA using the enzyme reverse transcriptase; and (3) amplification of the PCR product coupled to fluorescent detection of labeled PCR products (Vemula et al., 2016).

Real-time quantitative PCR (RT-qPCR) is a rapid, specific and sensitive TaqMan PCR method for detection, subgrouping and quantitation of pathogens. This assay increases the sensitivity of conventional PCR. A quantitative TaqMan PCR assay was once used to detect 175 nasopharyngeal aspirates obtained from children with respiratory symptoms in Hong Kong and it detected 36 RSV-positive samples, including 10 as RSV-A and 26 as RSV-B. In contrast, a cell culture-based assay only identified 21 and an immunofluorescence assay identified 32 RSV-positive specimens, all of which

were among those identified by the TaqMan PCR assay, suggesting the greater accuracy and sensitivity of the TaqMan PCR assay (A. Hu et al., 2003; N. Zhang et al., 2020).

3.2.2.Locked nucleic acid (LNA)-based one-tube nested real-time (OTNRT)-PCR

Locked nucleic acid (LNA)-based one-tube nested real-time (OTNRT)-PCR is an assay with very high sensitivity and low incidence of cross-contamination for detection of RSV (L. Zhao et al., 2019). A total of 143 nasopharyngeal aspirate samples that tested RSV-negative by qRT-PCR were confirmed as RSV-positive by sequencing the OTNRT-PCR products, (L. Zhao et al., 2019) indicating that OTNRT-PCR is more sensitive than RT-qPCR for detection of RSV in clinical samples (N. Zhang et al., 2020).

3.2.3.Reverse-transcription recombinase-aided amplification (RT-RAA)

Rapid reverse-transcription recombinase-aided amplification (RT-RAA) assay was developed as a molecular-based diagnostic method to detect subgroup RSV-A and B genomes in clinical specimens. This method mainly utilizes an enzyme mixture, including single-strand DNA binding protein (SSB), recombinase UvsX and DNA polymerase, to detect RNA amplicons of RSV (C. Chen et al., 2018). It is performed at 39°C in less than 30 minutes with high specificity (N. Zhang et al., 2020).

3.2.4.Reverse transcription strand invasion-based amplification (RT-SIBA)

Reverse transcription strand invasion-based amplification (RT-SIBA) is reverse transcription isothermal nucleic acid amplification for rapid detection of RSV with good sensitivity since it can detect as few as 10 copies of RSV RNA within 20 minutes (Eboigbodin et al., 2017). RT-SIBA does not need highly purified RNA for detection of RSV, which can reduce the complexity of specimen preparation and shorten the total detection cycle in clinical specimens (N. Zhang et al., 2020).

3.2.5.Simple Amplification-Based Assay (SAMBA)

SAMBA is a dipstick isothermal nucleic acid amplification approach, recently developed for the detection of HIV and influenza viruses. The approach involves a three-step procedure consisting of viral RNA extraction, target DNA amplification using an isothermal DNA polymerase and detection of the amplification product using a dipstick-based system. The SAMBA procedure takes approximately two hours to complete. Clinical performance of this approach has been evaluated for both seasonal and avian influenza viruses. While evaluating nasal/throat and nasopharyngeal swab specimens from 328 patients from the United Kingdom and Belgium, Wu et al. reported a sensitivity of 100% and 97.9%, respectively, for influenza A and B viruses compared to an RT-PCR approach (Wu et al., 2010). The analytical sensitivity using this approach was 95 and 85 copies of viral genomes for influenza A and B viruses, respectively. In another study, the same group had reported an

assay sensitivity of 95.3% with 99.4% specificity for the pH1N1 virus compared to a RT-PCR-based approach, based on testing RNA samples extracted from nasal/throat swab specimens from 262 patients (Wu et al., 2013).

3.2.6.Loop-Mediated Isothermal Amplification-Based Assay (LAMP)

LAMP is a DNA loop-mediated isothermal nucleic acid amplification approach that has been evaluated for detection of several viruses including severe acute respiratory syndrome (SARS) corona virus, rhinovirus, adenovirus, new castle disease virus, monkey pox virus, human immunodeficiency virus and influenza virus. Specific amplification of the target gene is determined by either photometrically detecting the magnesium pyrophosphate by-product released in the solution at the end of the reaction or by observing the color change following addition of SYBR green. Further details are provided in chapter 4.

The addition of RT in LAMP reaction (RT-LAMP) permits the amplification of RNA target (Fukuta et al., 2003), previously extracted from clinical specimens. Recently, for instance, Huang et al. (W. E. Huang et al., 2020) developed a rapid RT-LAMP assay for diagnosis of SARS-CoV-2 with LoD of 80 copies of viral RNA/ml in a sample within a 30 minutes reaction.

LAMP-based approaches have been successfully used for the detection of influenza viruses from clinical samples with sensitivity comparable to RT-PCR based assays. Using the LAMP-approach, Poon *et al*. reported 100% assay sensitivity for detection of seasonal influenza A viruses from subtypes H1N1

and H3N2 from clinical samples (Poon et al., 2005). The analytical sensitivity of the assay was 10 copies per reaction. Furthermore, during the 2009 H1N1 pandemic, a real-time reverse transcription LAMP-based assay (RT-LAMP) demonstrated a sensitivity of 97.8% with 100% specificity for the pandemic virus compared to an RT-PCR-based assay (Kubo et al., 2010). LAMP-based assays have also been successfully evaluated for the detection of highly pathogenic avian influenza A viruses from subtypes H5N1, H7N7 and H7N9, with sensitivities comparable to, or even higher than, RT-PCR-based approaches (M. Imai et al., 2006; Nakauchi et al., 2014). Parida et al. developed a RT-LAMP assay targeting the gene coding for HA for clinical diagnosis of the pH1N1 virus (Parida et al., 2011). The RT-LAMP assay performed better than WHO-approved RT-PCR assay while testing 239 acute-phase throat swab samples from patients with influenza-like illness. It demonstrated up to 10-fold higher sensitivity compared to a WHO-approved RT-PCR method with an analytical sensitivity of 0.1 $TCID_{50}$/mL (median tissue culture infective dose).

3.2.7.Nucleic Acid Sequence-Based Amplification (NASBA)

NASBA is an isothermal PCR-independent amplification method that uses a combination of three enzymes: avian myeloblastosis virus reverse transcriptase, RNAse H and T7 RNA polymerase in a single reaction. NASBA has been successfully evaluated for detection of both seasonal influenza A and highly pathogenic avian H5N1 and H7N9 avian influenza A viruses. Moore et al. evaluated NASBA for evaluation of H5N1 infection in 19 clinical samples obtained from confirmed cases of influenza A H5N1 infection in China. The

assay demonstrated an analytical sensitivity of 0.01 $TCID_{50}$ for A/Vietnam/1194/2004 H5N1 virus, demonstrating an assay sensitivity of 100% (Moore et al., 2004). In another study, Ge et al. used NASBA for rapid detection of the novel swine origin pH1N1 virus (Ge et al., 2010). In that study, NASBA demonstrated an assay sensitivity of approximately 50 copies per reaction, which was comparable or higher than that observed with a commercial swine origin influenza A virus (S-OIV) (H1N1) real-time RT-PCR kit and CDC TaqMan assay. Recently, Wang et al. developed a modified NASBA procedure, referred to as a simple method for amplifying RNA targets, or SMART, for detection of seasonal H1N1 and H3N2 and pH1N1 viruses (J. Wang et al., 2013). This isothermal amplification approach utilized single-stranded DNA (ssDNA) probes to serve as reporter molecules for capturing specific viral RNA (vRNA) sequences that are subsequently separated on a microfluidic chip under zero-flow conditions. The SMART assay demonstrated an analytical sensitivity of up to 10^5 vRNA copies/mL with an assay sensitivity of 98.3% and specificity of 95.7% for detection of influenza A viruses (Vemula et al., 2016).

3.2.8.Microarray-based approaches

Microarray-based approaches have proven to be useful tools for detection and subtyping of influenza viruses. For example, the FluChip microarray, a low-density DNA microarray, has been shown to detect H1N1, H3N2 and H5N1 strains in a few hours (Dawson et al., 2007; Townsend et al., 2006). A NanoChip 400 system (Nanogen Inc., San Diego, CA, USA) low-density microarray that employs one probe for the conserved gene coding for M and 97 probes for the cleavage site region of HA gene, was shown to be a useful

diagnostic tool for the H5N1 virus (Gall et al., 2009). Chiu et al. (Chiu et al., 2008) used DNA microarray for high-throughput multiplex detection of viruses in nasopharyngeal aspirate samples originated from children infected with respiratory viruses. The assay demonstrated overall sensitivity of 87% to 90% and specificity of ≥99% in the detection of RSV, influenza A virus and rhinovirus/enterovirus compared to RT-PCR.

However, all of these microarray-based assays require two or more enzymatic amplification steps of influenza viral RNA prior to hybridization. Moreover, detection requires labeling of multiple probes or incorporation of fluorescent dye- or biotin-conjugated nucleotides into double-stranded DNA (dsDNA) generated by RT-PCR. Furthermore, the sensitivities of most conventional microarray assays have been shown to rely on the efficiency of target amplification and hybridization of amplicons and probes. The multiple steps involved in these assays make them complex, expensive, labor intensive, susceptible to contamination and also make them prone to false negative results due to the presence of gene mutations, PCR inhibitors and RNA degradation. The design of multiple, virus-specific primer sets and assay optimization procedures pose diverse challenges (J. Zhao et al., 2015).

3.2.9.Nucleic acid sequencing approaches

Sanger sequencing, also referred to as the dideoxy procedure, is a chain termination method of DNA sequencing. Developed by Fredrick Sanger and colleagues in the late 1970s, this approach involves the use of DNA polymerase, a pair of DNA primers, unlabeled deoxynucleotide triphosphates (dNTPs) and chain-terminating dideoxynucleotides (di-ddNTPs) with each

base labeled with a unique fluorophore. Sanger sequencing has been widely used for whole genome sequencing of influenza viruses. Moreover, Sanger sequencing has also been used for the detection of antiviral resistance among circulating influenza viruses (Vemula et al., 2016).

Next-Generation Sequencing (NGS) offers significant improvements in sequencing speed and throughput when compared with Sanger sequencing. Since NGS directly analyzes nucleic acid fragments extracted from samples, it obviates the complex and time-consuming vector cloning imperative to Sanger sequencing. An added merit of NGS is that of the sequencing cost even if is expected to decrease further in the near future.

Several companies produce different NGS machines that use different methods of sequencing, reagents and data analysis tools (Vemula et al., 2016). For example, pyrosequencing (Roche 454) detects release of pyrophosphate following incorporation of nucleotides in a DNA polymerization process.

Bright and colleagues were the first to use this platform to study the incidence of adamantine resistance among seasonal influenza A viruses from subtypes H1N1, H1N2 and H3N2, isolated worldwide from 1994–2005 (Bright et al., 2005). Since then, several groups have successfully used the pyrosequencing platform to identify mutations in genes coding for M2 and NA responsible for resistance to adamantanes, amantadine and rimantadine and oseltamavir, respectively, among seasonal and highly pathogenic H5N1 avian influenza viruses. Pyrosequencing has also been used by a few groups to identify SNPs in the gene coding for hemagglutinin (H. K. Lee et al., 2014; M. Levine et al., 2011).

Illumina's NGS platforms detect release of fluorescent labels from incorporated nucleotides in a DNA polymerization process (Reta et al., 2020). Using an Illumina MiSeq sequencer (San Diego, CA, USA), many groups acquired complete genome sequence information from influenza virus isolates (Greninger et al., 2010; Kuroda et al., 2010; Ren et al., 2013; Rutvisuttinunt et al., 2013; Whitehead et al., 2012).

There are a few alternative NGS platforms, besides the Roche 454 and Illumina MiSeq platforms, that may be suitable for influenza diagnosis, including Life Technologies/Applied Biosystem's Ion personal genome machine (PGM), Ion Proton and SOLiD NGS platforms and Pacific Biosciences' PACBIO RS/RSII single-molecule, real-time sequencing platform. Each platform has its own advantages and disadvantages, as the underlying proprietary sequencing techniques are quite different.

Recently, a high-throughput whole genome sequencing (WGS) method with the Oxford nanopore MinION portable sequencer was developed to test influenza A and B viruses, as subsequently validated by the Illumina MiSeq platform. The overall accuracy, precision, as well as recall rates, were 99.95%, 97.88% and 89.41%, respectively, from 1D reads and 99.97%, 99.86% and 93.28%, respectively, from $1D^2$ reads (K. Imai et al., 2018).

Kustin et al. (Kustin et al., 2019) used NGS for rapid and robust identification of respiratory viruses in clinical samples. It was applied to track influenza A (H1N1) pdm09 virus (Baillie et al., 2012).

NGS will play an increasing role in identification of RSV and characterization of strain diversity in special cases (Grad et al., 2014). The public health role will include identification of evolutionary diversity and potential patterns of

transmission while using primary clinical specimens (Agoti et al., 2015). Also, apart from diagnostic roles, NGS of RSV has helped identify mutation rates for RSV-A and -B as well as global circulation patterns of RSV-A and -B clades (Agoti et al., 2015). SNP analysis of RSV is indicating a significant number of SNPs in RSV-G and -F genes, while the P gene of RSV is more conserved (Grad et al., 2014; Griffiths et al., 2017).

Unlike PCR and DNA microarray methods, NGS does not require prior knowledge of genomic sequences of the viral pathogens. It does not also require target specific PCR primers and oligonucleotide probes (Kustin et al., 2019; Zhen Lin et al., 2014). However, the use of NGS in clinical laboratories is limited because of the following reasons: the turnaround time, the number of samples per run, cost of sequencers and requirement of skills in bioinformatics (Dessilly et al., 2018; Jerome et al., 2019; Souf, 2016).

3.3. Point of Care (POC) tests

Point-of-care testing (POCT or bedside testing) is defined as medical diagnostic testing at or near the point of care—that is, at the time and place of patient care (Quesada-González & Merkoçi, 2018).

Rapid Influenza Diagnostic Tests (RIDTs) are antigen-based tests developed for rapid diagnosis of influenza virus infections in POC settings. These tests use monoclonal antibodies that target the viral nucleoprotein and employ either enzyme immunoassay or immunochromatographic (lateral flow) techniques.

Available in dipstick, cassette, or card formats, RIDTs can be completed in less than 30 min, with the results observed visually based on a colour change

or other optical signals. Due to simplicity in their use and the speed of obtaining assay results, RIDTs are commonly used for the diagnosis of influenza infections. Several FDA-approved RIDTs are currently available on the market. Most of these tests can either detect or distinguish influenza A and B viruses, detect only influenza A viruses, or both influenza A and B viruses (but cannot discriminate influenza A and B). However, none of the RIDTs can distinguish between the different influenza A subtypes Performance of RIDTs is dependent on the prevalence of circulating influenza viruses in the population (Cruz et al., 2010; Harper et al., 2009). During peak influenza activity, positive predictive values are high and false positives are, therefore, likely to be observed. However, during low influenza prevalence, negative predictive values are high, with low positive predictive values. For diagnosis of seasonal influenza infections, RIDTs have demonstrated variable assay performance with sensitivities ranging between 10%–70%, with up to 90% specificity compared to standard RT-PCR-based assays. Performance of RIDTs have been shown to be better in children compared with adults (approximately 13% higher), potentially due to higher viral loads and longer viral shedding in children compared with adults (Cruz et al., 2010; Drexler et al., 2009; Louie et al., 2010). Moreover, due to a high rate of false negatives, the CDC advised physicians not to discontinue antiviral therapy despite negative RIDT results. The main disadvantage of these tests is that for detection of influenza viruses, RIDTs have demonstrated lower sensitivity compared RT-PCR-based approaches. Although RIDT have demonstrated variable sensitivity, they still remain the test of choice in most clinical virology

laboratories around the world due to the speed in obtaining results, simplicity in assay procedure and cost.

For RSV rapid assays are generally available in three formats, immunochromatographic (ICR) tests, enzyme immunoassays (EIAs) and optical immunoassays (OIAs) and most assays target the RSV-F surface glycoprotein (Prendergast & Papenburg, 2013) and nucleocapsid proteins of RSV (P. Walsh et al., 2014).

Recent antigen detection tests utilize specialized detection approaches to improve test characteristics. As shown in Table 1, these tests have improved sensitivities and specificities compared to molecular assays. For example, the Sofia RSV assay has a sensitivity and specificity of 78.6% and 93.9%, respectively, compared to molecular assays (Tuttle et al., 2015) (Table 1). This fluorescence immunoassay uses a virus disruption step prior to detection of viral nucleoproteins and is designed to detect RSV from nasopharyngeal swabs and aspirates from pediatric patients less than 19 years of age but not from adults or immunocompromised patients. As shown in Table 1, these improvements in POC test characteristics are not kit dependent and similar improved sensitivities and specificities are seen with the BD Veritor RSV POC assay, which has a sensitivity of 81.6% and a specificity of 99.1% (Schwartz et al., 2015). This chromatographic assay, which detects the RSV-F glycoprotein, is intended for use on nasopharyngeal washes, aspirates and swabs from patients under 20 years of age. With both described second-generation POC tests, negative specimens should be verified with another method (e.g., viral culture or an FDA-approved molecular test) according to the manufacturer's instructions. It should be noted that both described systems have reduced

sensitivities for RSV-A and -B compared to real-time PCR assays (Kanwar et al., 2015). Furthermore, compared to culture and some molecular tests, these second-generation POC assays suffer from the inability to distinguish RSV-A from RSV-B (Griffiths et al., 2017).

4. LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP)

In 2000 Notomi et al. firstly described a new technique for the isothermal amplification of nucleic acids, named Loop-mediated isothermal AMPlification (LAMP).

This technology is an innovative non-PCR based nucleic acid amplification method performed using a strand-displacement polymerase under isothermal conditions. Both amplification and detection of nucleic acid sequences can be completed in a single step by incubating the mixture of sample, primers, DNA polymerase at a constant temperature, allowing the use of simple and cost effective reaction equipment (Notomi et al., 2000).

The amplification efficiency of LAMP is very high and the reaction proceeds rapidly as there is no need for initial heat denaturation of the template DNA or thermal cycling.

LAMP has been explored so far in hundreds of papers for the detection of DNA of many different organisms, such as bacteria (Aryan et al., 2010; W. Yang et al., 2009) viruses (Curtis et al., 2009; Kaneko et al., 2005; Thai et al., 2004; Yoda et al., 2009), fungi (Sun et al., 2010; Uemura et al., 2008), parasites (J.-H. Chen et al., 2010; Nkouawa et al., 2009; H. Zhang et al., 2009) and it is also suitable for the analysis of human DNA (Minnucci et al., 2012). Thanks to

its peculiar characteristics, LAMP has shown to be an easy and rapid diagnostic tool in molecular medicine.

Moreover, a Reverse Transcription (RT) LAMP has been developed for the detection and amplification of RNA by introducing a reverse transcriptase in the reaction mix, expanding the fields of applications for this technique (Parida et al., 2004; Ushio et al., 2005).

Thus, LAMP constitutes an attractive alternative to PCR for sequence detection, with a sensitivity and quantitative performance comparable to PCR, allowing different detection methods, simplex and multiplex reaction applications in a fast, easy and potentially cheap molecular diagnostics platform (Parida et al., 2008).

4.1. LAMP principle

LAMP amplification method relies on the use of a thermostable DNA polymerase with strand displacement activity (*Bst* polymerase from *Bacillus stearothermophilus*) and four primers specifically designed to recognize six distinct regions on the target gene. F3 and B3 are the most external primers, which allow the strand-displacement activity of the enzyme at the beginning of the reaction. The inner primers are FIP (Forward Inner Primer) and BIP (Backward Inner Primer).

Both inner primers contain two distinct sequences (F2 + F1c for the FIP primer and B2 + B1c for the BIP primer), corresponding to the sense and antisense sequences of the target DNA, one for the initial priming and the other for the subsequent self-priming (Figure 8).

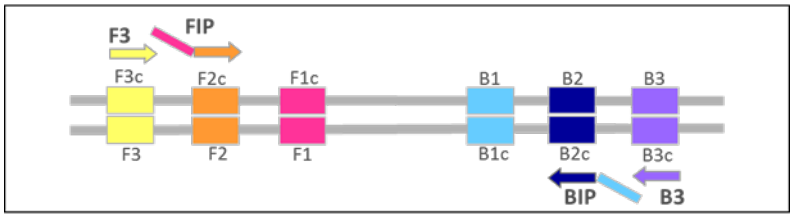


Figure 8. Schematic representation of the four primers, recognizing six distinct nucleotide sequences on the target gene, required for a basic LAMP reaction: two internal primers (FIP, BIP) and two external primers (F3, B3).

The mechanism of the LAMP amplification reaction includes three steps (Figure 9):

I. production of starting structure;

II. cycling amplification;

III. elongation and recycling.

All four primers are operative in the initial steps of the reaction, but in the later cycling steps, only the inner primers are required for strand displacement DNA synthesis.

At a constant reaction temperature, inner primer FIP hybridizes to F2c in the target DNA and initiates complementary strand synthesis (structure 1, Figure 9). Outer primer F3 hybridizes to F3c in the target and initiates strand displacement of the new DNA chain (structure 2, Figure 9), releasing a FIP-linked complementary strand, which forms a looped-out structure at one end (structure 4, Figure 9). This single stranded DNA serves as template for BIP-initiated DNA synthesis and subsequent B3-primed strand displacement DNA synthesis leading to the production of a dumbbell form DNA which is quickly converted to a stem-loop DNA (structure 6, Figure 9).

The dumbbell serves as the starting structure for LAMP exponential amplification, the second step of the LAMP reaction. The stem-loop DNA

structure contains a double stem-loop which is the starting point for the whole LAMP amplification process as it promotes an amplification from its self-annealed 3' terminus and from a newly annealed internal primer (FIP or BIP) (structure 7, Figure 9). During amplification, FIP hybridizes to the loop in the stem-loop DNA and primes strand displacement DNA synthesis, generating as an intermediate one gapped stem loop DNA with an additional inverted copy of the target sequence in the stem and a loop formed at the opposite end via the BIP sequence (structure 8, Figure 9). Subsequent self-primed strand displacement DNA synthesis yields one complementary structure of the original stem-loop DNA and one gap repaired stem-loop DNA (structure 10, Figure 9) with a stem elongated to twice as long and a loop at the opposite end (structure 9, Figure 9).

Both of these products then serve as templates for BIP-primed strand displacement in the subsequent cycles and in the elongation and recycling step. Thus, in LAMP the target sequence is amplified 3-fold every half cycle.

Amplification proceeds promoting itself, each strand being displaced by elongation of the new loops formed (Notomi et al., 2000).

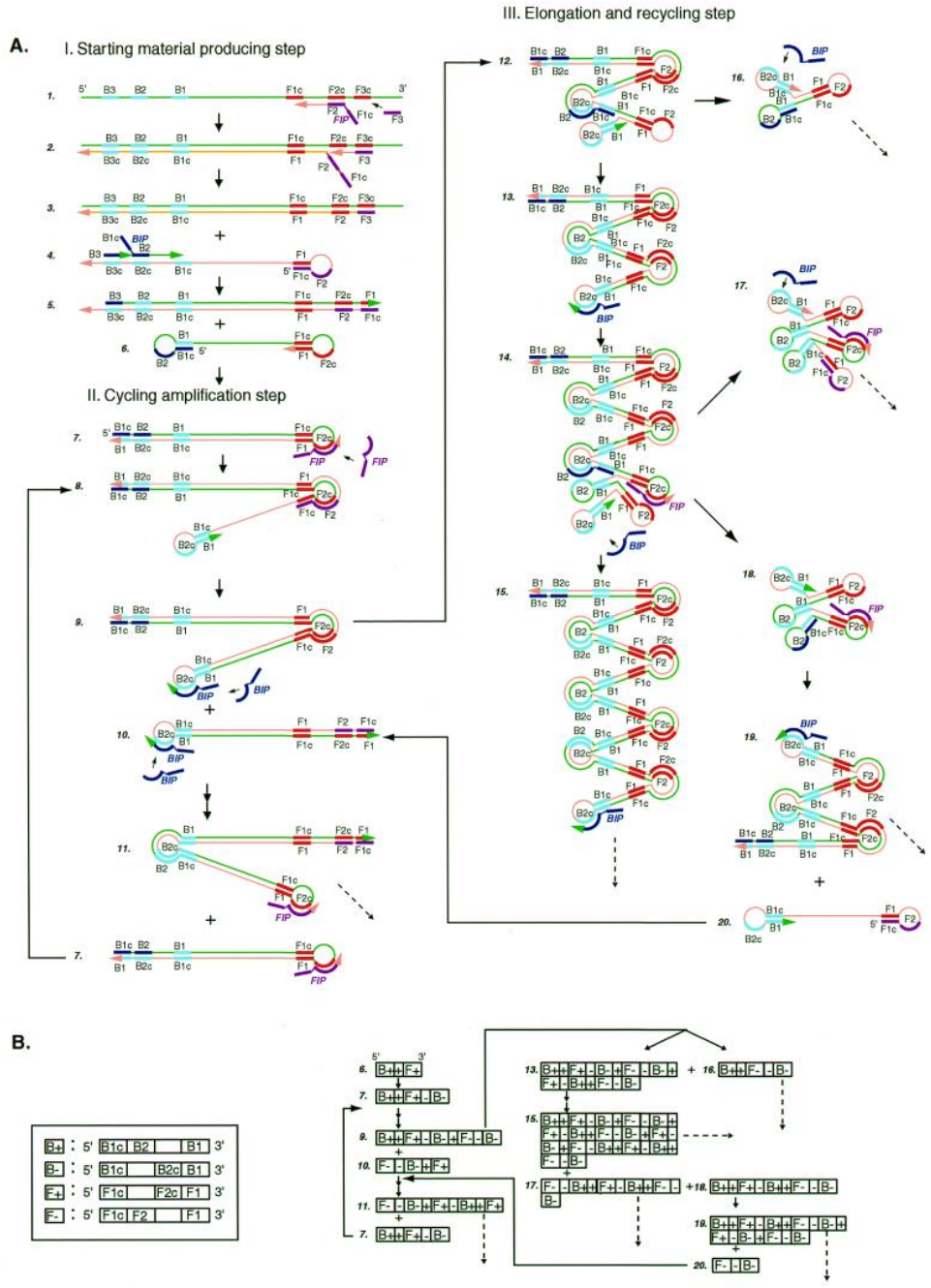


Figure 9. Schematic representation of the steps of a LAMP reaction. This figure shows the process that starts from primer FIP. DNA synthesis can also begin from primer BIP.

The final product is a mixture of stem-loop DNA with various stem length and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target sequence in the same strand (Figure 10).

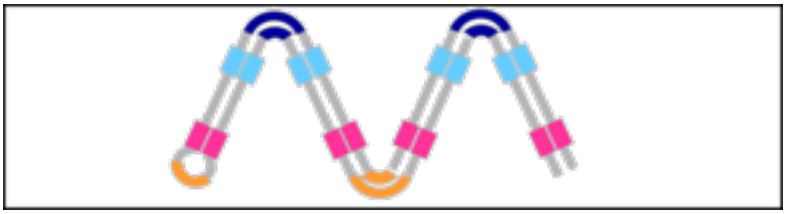


Figure 10. Schematic representation of inverted repeats concatamers that are the main amplification products of a LAMP reaction.

4.2. Loop primers

In order to accelerate the LAMP reaction, it is possible to introduce additional primers, called Loop Primers.

The Loop Primers LF (Loop Primer Forward) or LB (Loop Primer Backward) contain sequences complementary to the single stranded loop region (either between the F1 and F2 regions or between the B1 and B2 regions) on the 5' end of the dumbbell-like structure, providing increased number of starting points for DNA synthesis.

In particular, two different types of dumbbell-like structures are generated during a LAMP reaction:

i. a dumbbell on which the FIP primer and the LB primer (if present) hybridize: the LB primer anneals on the stem-loop structure generated by the self-annealing of the BIP primer, while the FIP primer hybridizes to the complementary sequence on the second stem-loop structure of the dumbbell;

ii. a dumbbell on which the BIP primer and the LF primer (if present) hybridize: the LF primer anneals on the stem-loop structure generated by the self-annealing of the FIP primer, while the BIP primer hybridizes to the complementary sequence on the second stem-loop structure of the dumbbell.

As a consequence, each loop primer will hybridize to the stem-loop that is not hybridized by the inner primer and will prime strand displacement DNA synthesis (

Figure 11).

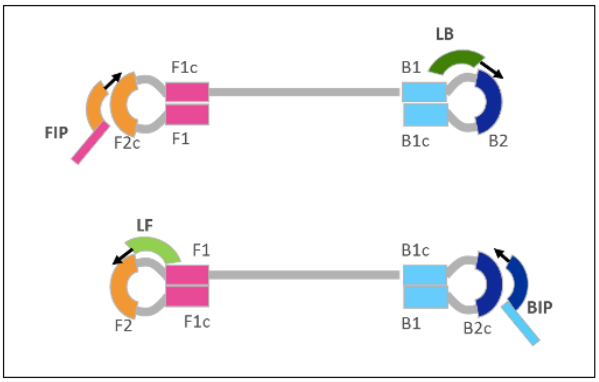


Figure 11. Schematic representation of the two stem-loop structures (also called "dumbbell" structures) generated during a LAMP reaction: FIP and LB primers will hybridize on one dumbbell type, while BIP and LF primers will hybridize on the other dumbbell type.

The loop primers act as catalyzers of the amplification reaction, since their presence increases the rate of DNA production and therefore the sensitivity of the method, considerably decreasing the reaction time of the original LAMP method (Nagamine et al., 2002).

By these peculiar amplification dynamics and exploiting the continuous displacement by *Bst* polymerase of the already produced DNA strands with no need for denaturation steps, it is possible to amplify the initial DNA amount up to 10^{10} times in less than 60 minutes with very high specificity, due to the many independent target-recognition events.

4.3. Different forms of LAMP assays

4.3.1.Conventional LAMP

LAMP was successfully demonstrated to detect human respiratory pathogens, such as *Mycobacterium tuberculosis* (Bentaleb et al., 2016; P. Kumar et al., 2014), *Streptococcus pneumoniae* (Seki et al., 2005; Takano et al., 2019; Xia et al., 2014), *Bordetella pertussis* (Fujino et al., 2015; Kamachi et al., 2017) and carbapenem-resistant *Klebsiella pneumoniae* (Liao et al., 2020; Nakano et al., 2015). Furthermore, LAMP was also devised to diagnose pathogens associated with food-borne diseases, for instance, *Salmonella typhi* (Abdullah et al., 2014; F. Fan et al., 2015) (Abdullah et al. 2014; Fan et al. 2015), *Campylobacter jejuni* and *Campylobacter coli* (Babu et al., 2020; Pham et al., 2015) and *Helicobacter pylori* (Bakhtiari et al., 2016; Horiuchi et al., 2019). Other successful examples of LAMP usage include *Neisseria meningitidis* (S.-J. Fan et al., 2020; D. Lee et al., 2016), *Listeria monocytogenes* (Tirloni et al., 2017; Ye et al., 2015), *Entamoeba histolytica* (Foo et al., 2017; Rivera & Ong, 2013) and human leptospirosis (Seesom et al., 2015; Varsha et al., 2020). LAMP was also efficiently formulated against several multidrug-

resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Kim et al., 2016). The high LAMP assay usage proves its potential as a means for accurate molecular detection and to differentiate human pathogens.

LAMP was also used for the detection of a wide range of viruses. For example, *adenovirus* (Ziros et al., 2015), *varicella zoster* (Higashimoto et al., 2019; Niihara et al., 2017) and *cytomegalovirus* (X. Wang et al., 2015). *Hepatoviruses* such as hepatitis B (Quoc et al., 2018) can also be detected using LAMP technique. The results showed that LAMP assay is promising for hepatitis diagnostics, donor screening, epidemiological studies and therapeutic monitoring of patients undergoing antiviral treatment.

Protozoan parasites are equally harmful to humans and are one of the leading causes of death in the world. A parasitic disease detected by using LAMP was for malaria diagnosis of *Plasmodium* spp. (Britton et al., 2016; Mohon et al., 2019; Y. Zhang et al., 2017).

There was a number of other reported significant practices of LAMP including *Trichomonas vaginalis* (Adao & Rivera, 2016; Li et al., 2020) and it was shown that LAMP is at least 1000 times more sensitive than the conventional PCR.

The routine detection of fungi is laborious and expensive. Different studies were carried out to detect an extensive range of fungi, including *Candida albicans* (Fallahi et al., 2020). The assay can be efficiently used to detect fungi from the environment (Nakayama et al., 2017). The real-time LAMP assay established could be a promising assay for early diagnosis of *Aspergillus* infection in humans (Tone et al., 2019).

Other successful examples of LAMP usage to detect fungal infections include *Fonsecaea* species (Sun et al., 2010), *Trichosporon asahii* (J. Zhou et al., 2015) and *Pneumocystis jirovecii* (Scharmann et al., 2020). LAMP requires minute quantities of DNA for detection of the fungal pathogen with unrivalled high sensitivity and easy handling. These qualities make LAMP suitable for all ranges of applications in the diagnosis of communicable diseases.

4.3.2.RT-LAMP

Throughout the years, conventional LAMP method has been modified and implemented.

Reverse transcription loop-mediated isothermal amplification (RT-LAMP) is a one-step nucleic acid amplification technique used by taking a step further and adding LAMP with a reverse transcriptase enzyme to allow RNA detection. Like RT-PCR, RT-LAMP uses reverse transcriptase to make complementary DNA from RNA and is further amplified by using DNA polymerase.

This method is valuable and beneficial because the assay can be completed in a single step, by incubating all the primers and enzymes (polymerase and reverse transcriptase) with a constant temperature (Mori et al., 2013; Notomi et al., 2000, 2015) and this was very effective in detecting viruses with an RNA genome.

RT-LAMP was established for Dengue virus (S. Hu et al., 2015; Lau et al., 2015; Y. Zhou et al., 2019), influenza viruses (Le Thi et al., 2020; Nakauchi et al., 2014), Hepatitis C (C. Yang et al., 2019; N. Zhao et al., 2017), Ebola virus (Bonney et al., 2020), respiratory syncytial virus (Hoos et al., 2017; Y. Hu et

al., 2019), zika virus (L. E. Lamb, Bartolone, & Chancellor, 2020), Middle East Respiratory Syndrome coronavirus (Mers-CoV) (Bhadra et al., 2015; S. H. Lee et al., 2016) and more recently severe acute respiratory syndrome coronavirus 2 (Sars-CoV2) (Dao Thi et al., 2020; X. Hu et al., 2020; W. E. Huang et al., 2020; Kashir & Yaqinuddin, 2020; Kitagawa et al., 2020; L. E. Lamb, Bartolone, Ward, et al., 2020; Lu et al., 2020; Pezzi et al., 2020). Apart from helping in diagnosis, the RT-LAMP can also be used as an epidemiologic surveillance system for human virus infections.

Currently, one of the most successful uses of RT-LAMP is diagnosing the HIV retrovirus. RT-LAMP was developed to diagnose dormant HIV stage in infected individuals (Curtis et al., 2018; Ocwieja et al., 2015).

RT-LAMP was also used to detect plant pathogens. One of the recent examples of RT-LAMP plant pathogens detection is Apple chlorotic leaf spot virus (ACLSV), by targeting the virus coat protein gene. The reaction conditions were optimized according to temperature and reaction time. The RT-LAMP developed was capable to detect concentration as low as 0.02 μg/μL). at 64°C. The study reported that RT-LAMP has a 100-fold greater sensitivity as compared with RT-PCR (2.29 μg/μL). The assay could be successfully applied to field-collected apples to diagnose the diseases and to potentially control the spreading of ACLSV (Peng et al., 2017).

RT-LAMP was as well developed for animal pathogens, for example, *Batai virus* (BATV), a mosquito-borne virus, which would cause congenital defects in ruminants (cattle) (Liu et al., 2016).

Importantly, RT Q-LAMP (“Reverse Transcription-Quenching Loop-mediated Isothermal Amplification”) technique, developed by DiaSorin, has

represented a further improvement of the 'traditional' LAMP reaction (Spinelli et al., 2015). The insertion of fluorescent probes and a new polymerase with both reverse-transcriptase and DNA polymerase activities has allowed the amplification and the real-time detection of multiple target genes in a single tube, with a single enzyme, at a single temperature.

4.3.3.Electric LAMP

A study reported by Salinas and Little (Salinas & Little, 2012) explains on the development of electric LAMP (eLAMP), an electronic simulation where it provides a fast and inexpensive putative tests of LAMP primers on target sequences compatibility. This method helps to improve the efficiency and aids to determine the opportunity of using existing primers to detect recently discovered sequence variants. In this study, sets of existing LAMP primers were tested using this eLAMP on 40 whole-genome sequences of *Staphylococcus*. The results correctly predicted that the tested primer sets would amplify from *Staphylococcus aureus* genomes and not from other *Staphylococcus* species.

4.3.4.In-disc LAMP

Santiago-Felipe et al. (Santiago-Felipe et al., 2016) developed an assay for detection of pathogenic *Salmonella* spp. and identification of bovine meat in meat samples by using the principle of in-disc LAMP (iD-LAMP) and quantitative optical read-out by a disc drive. iD-LAMP is an integrated device composed of micro-reactors embedded onto compact discs for real-time

targeted DNA determination. Similar reagents used in conventional LAMP were used in this iD-LAMP except that it was performed in a micro-reactor placed in a 65°C oven. During the incubation, the disc was cyclically scanned and a sample was considered positive when the optical response was greater than the cut-off value. This study shows that the result of iD-LAMP was comparable to those obtained by conventional LAMP with an extra advantage of using a smaller amount of template.

4.4. Detection of LAMP amplification products

Two principal methods have been widely used to detect LAMP amplification products and distinguish between positive and negative samples: turbidimetry or fluorescence.

Turbidimetry detection is made possible by the high amplification efficiency of LAMP reaction (Mori et al., 2001, 2004). This detection method consists in the measurement of turbidity of the reaction mix, due to the precipitation of magnesium pyrophosphate. This insoluble salt is formed by the interaction between Mg_{2+} present in solution and the inorganic pyrophosphate produced by the incorporation of dNTPs in the DNA growing strands. Thus, the amount of magnesium pyrophosphate is proportional to the amount of amplified DNA. The turbidity is visible by the naked eye and is measurable on a turbidimeter as a transmittance signal either at end-point for a qualitative analysis or in real-time, allowing quantitative applications.

Another approach for the detection of LAMP reaction products is represented by the use of fluorescent intercalating dyes that bind double-stranded DNA: they allow a real-time direct visualization of the isothermal

amplification by measuring the increase in fluorescence throughout the reaction correlated to the exponential synthesis of LAMP amplicons. The detection step has then to be followed by an annealing analysis to confirm the reaction product.

However, turbidimetry and fluorescent intercalating dyes are not very specific detection approaches, because they allow detecting double–stranded DNA without easy and rapid discrimination between specific and non-specific products.

Other fluorescence approaches are being applied to LAMP technique, such as molecular beacons or quenching probes, relying on sequence-specific detection of a desired LAMP product. The use of fluorescent–labelled target-specific probes results in increased specificity and sensitivity. In addition, the use of different fluorophores specific for distinct targets allows to potentially amplify many sequences simultaneously. This was applied in conventional LAMP reaction as an advancement of the method, namely multiplex LAMP (mLAMP) (Wong et al., 2018).

4.5. Advantages of LAMP

As suggested by WHO, the criteria for an ideal diagnostic test must comprise sensitivity, specificity, low-cost, simplicity, rapidity, adaptability to all kinds of climatic changes and the availability of instruments (Mabey et al., 2004). Since detection of diseases is becoming progressively more difficult due to the occurrence of new as well as existing disease or pathogens, an alternative diagnostic method is in need to be discovered. Ever since its

development, the striking properties of LAMP have encouraged researchers to further explore its usage for analysis in various fields.

The primary characteristic of LAMP is the ability to amplify nucleic acids at isothermal conditions, allowing the use of simple and cost-effective reaction equipment.

The use of a polymerase with strand-displacement activity increases exponentially the speed of reaction, unbinding the amplification to the extension step, as instead happens for PCR. Unlike any other nucleic acid amplification technique, for example, PCR, LAMP could be performed in just 30 min rather than at least 90 min for PCR. In addition, a LAMP amplification proceeds rapidly since the initial heat denaturation for the DNA template is not required (Wong et al., 2018).

This high efficiency of LAMP reaction also determines a deep sensitivity, comparable to that of nested PCR.

Its simplicity eliminates the need of advanced instruments as needed in other amplification technique. LAMP has the potential to improve molecular biology technology with the simplicity of the assay as it is much easier to use as compared with PCR. The simplicity of LAMP can also be described in the method of analysing the results. Thus, unlike PCR, tedious gel electrophoresis procedure of the amplification products is not needed (Wong et al., 2018).

Moreover, LAMP can be made into a portable device to ease transportation to the site of diagnosis, for example, the diagnosis of the Ebola virus and food-borne pathogens (Kurosaki et al., 2016; Safavieh et al., 2016; Uddin et al., 2015), especially in developing countries due to its ease of performance without needing any advanced equipment or experts to operate it.

LAMP assays also appeared unaffected by the presence of nontarget DNA in the acquired samples (Kaneko et al., 2007; Zhibing Lin et al., 2012) which resembles its high specificity characteristic. The LAMP assay is stable against some PCR inhibitors such as blood and detection can be performed without the template extraction step and with a non-processed sample (Francois et al., 2011; Mori et al., 2013; Nagamine et al., 2001; Q. Wang et al., 2013).

Importantly, the coupling of RT and amplification step, together with the real-time detection, simplifies strongly the diagnostic workup, introducing a one-step method, more reliable and with lower risk of cross-contamination compared to the multi-step procedure of PCR, resulting in an overall simplification of the diagnostic procedure.

Furthermore, the use of labelled oligonucleotide probes specific for the transcripts of interest has allowed the development of a LAMP multiplex technology, in which more than one target gene is amplified within the same reaction tube with very high efficiency. For example, it is possible to perform duplex and triplex reactions, in which respectively two or three genes of interest are amplified at the same time and real-time monitored, by the use of fluorophores emitting at different wavelengths revealed by dedicated fluorescence channels.

Finally, the possibility to simultaneously amplify an internal control within the same reaction tube represents a further advantage of this technique. The internal control could consist in a housekeeping gene (ex: GUSβ, ABL, etc.) or in an exogenous nucleic acids. Its amplification allows verifying the proper extraction of RNA from virus, the quality and integrity of the extracted RNA, as well as the correct reaction conditions (buffer, enzyme, temperature, etc.).

All these characteristics have made LAMP a very useful method for the detection of many different organisms, such as bacteria, viruses, fungi and parasites.

AIM OF THE STUDY

Influenza is an infectious respiratory disease that, in humans, is caused by three immunologic types (A, B and C) of RNA viruses within the *Orthomyxoviridae* family. The WHO estimates that annual epidemics of influenza result in ~1 billion infections, 3–5 million cases of severe illness and 300,000–500,000 deaths (Krammer et al., 2018). Influenza A is further described by two viral proteins expressed on its surface, hemagglutinin and neuraminidase. Seasonal influenza is typically caused by viruses that contain one of three major subtypes of hemagglutinin (H1, H2, or H3) and one of two subtypes of neuraminidase (N1 or N2). Influenza B is not classified into subtypes (Centers for Disease Control and Prevention (CDC), 2020c).

Influenza is highly contagious and can take on a variety of appearances, ranging from isolated respiratory findings that resemble the common cold, to severe pneumonia requiring hospitalization. Persons at higher risk for hospitalization from seasonal influenza include children <2 years of age, adults >65 years of age and those with significant comorbidities (Centers for Disease Control and Prevention (CDC), 2020a; Gaitonde et al., 2019).

Although characterized by annual seasonal epidemics, sporadic and unpredictable global pandemic outbreaks also occur that involve influenza A virus strains of zoonotic origin. Pandemic influenza occurs every 10–50 years and is characterized by the introduction of a new influenza A virus strain that is antigenically very different from previously circulating strains; the lack of pre-existing immunity in humans is often associated with the severity of the infection and an increase in mortality (Krammer et al., 2018).

RSV infection is more prevalent in infants and toddlers and is a leading cause of hospitalization in this age group, but RSV also causes disease in adults that can be severe in certain populations (Centers for Disease Control and Prevention (CDC), 2020a). In infants and young children, RSV disease can range from a cold-like illness, bronchitis, or croup, to lower respiratory infections such as bronchiolitis and pneumonia. In adults, symptomatic infection usually presents as an upper respiratory tract illness with runny nose (rhinorrhea), sore throat (pharyngitis) and cough, with some patients also complaining of headache, fatigue and fever. High-risk adults, such as those with certain chronic illnesses or immunosuppression, may have more severe disease, such as pneumonia (Centers for Disease Control and Prevention (CDC), 2020b).

During epidemics, despite good accuracy of diagnosis based on clinical presentation, rapid and accurate diagnostic tests are required to discriminate and/or confirm virus-specific infection, mitigating the spread of virus within a community and facilitating immediate treatment (S. Kumar & Henrickson, 2012). Moreover, laboratory testing may be useful in hospitalized patients with suspected influenza and in patients for whom a confirmed diagnosis will change treatment decisions.

Importantly, rapid molecular assays are the preferred diagnostic tests because treatment with one of four approved anti-influenza drugs may be considered if the patient presents within 48 hours of symptom onset. The benefit of treatment is greatest when antiviral therapy is started within 24 hours of symptom onset (Gaitonde et al., 2019).

In recent years, thanks to the availability of technological platforms and instrumentations, PCR-based molecular assays are used in clinical practice (Merckx et al., 2017). Processing biological samples is required to extract viral genomes to be amplified by multiplex PCR or RT-PCR and this process is time consuming, needing skilled staff and equipped laboratories.

LAMP (Loop Mediated Isothermal AMPlification) can solve several of these problems as it is faster, precise, sensitive and specific. It is an innovative non-PCR based nucleic acid amplification method performed using a strand-displacement polymerase under isothermal conditions used for rapid detection of specific genes (Cai et al., 2008; J. Huang et al., 2012; Ito et al., 2006; Kaneko et al., 2005; Notomi et al., 2000). Several aspects of the LAMP reaction make this technology advantageous. First, only a single type of enzyme is required and the amplification can be carried out at a constant temperature. Second, the fundamental characteristics of the inner primer provide the amplification with a specificity that is much greater than the one observed in PCR methods and the sensitivity is similar to that of nested PCR.

Moreover, the rapid, sequential progression of the LAMP amplification reaction contributes to its high amplification efficiency and small quantities of a gene can be amplified within a short time (Notomi et al., 2015).

Importantly, LAMP method combined with reverse transcription (called RT-LAMP) is a method to simultaneously obtain cDNA from template RNA and amplify DNA (Ahn et al., 2019).

Taking into consideration all these aspects, the ultimate aim of this thesis is to develop a RT Q-LAMP-based assay in a multiplex strategy, targeting conserved regions of influenza A, B and RSV viruses to differentially detect

and discriminate these infections. Both reverse-transcription and amplification are performed in a single-step from unprocessed clinical specimens and optimized using reagents with improved sensitivity and specificity, allowing clinicians to obtain results for a therapeutic intervention in less than 60 minutes.

MATERIALS AND METHODS

5. DEFINITION OF THE REACTION STRATEGY

According to the project's aim, we planned to design four different RT Q-LAMP primer sets to be used in multiplex strategy:

- the first set targeting influenza A H1N1 and H3N2 viruses, labelled with FAM fluorophore and detected in 500 nm channel of Liaison MDX instrument
- the second set targeting influenza B viruses, labelled with JOE fluorophore and detected in 560 nm channel
- the third set targeting RSV-A and RSV-B, labelled with Cal Fluor red 610 (CFR610) fluorophore and detected in 610 nm channel
- the fourth set detecting and amplifying an Internal Control (IC) target, labelled with Quasar 670 (Q670) fluorophore and detected in 682 nm channel.

If the influenza A, B and RSV are 0 for a sample and the IC (682 nm) Tt is non-zero and ≤60, the result is reported as "Not Detected" for all three viruses. If the influenza A (500 nm channel) Tt value is ≤60 for a sample, the result is reported as "Detected" for influenza A RNA. The IC is not applicable. If the influenza B (560 nm channel) Tt value is ≤60 for a sample, the result is reported as "Detected" for influenza B RNA. The IC is not applicable. If the RSV (610 nm channel) Tt value is ≤60 for a sample, the result is reported as "Detected" for RSV RNA. The IC is not applicable. If the influenza A, B and RSV are 0 for a sample and the IC Tt is also 0, the result is reported as "Invalid."

This specimen should be re-assayed. Table 2 shows the diagnostic algorithm of the assay:

Influenza A (Tt value)	Influenza B (Tt value)	RSV (Tt value)	IC (Tt value)	Interpretation
0	0	0	≤ 60; ≠ 0	Viruses not detected
≤ 60; ≠ 0	0	0	N/A	Influenza A detected
0	≤ 60; ≠ 0	0	N/A	Influenza B detected
0	0	≤ 60; ≠ 0	N/A	RSV detected
0	0	0	0	Invalid, re-assay

Table 2. Diagnostic algorithm of the assay.

6. INFLUENZA A, B AND RSV SEQUENCES SELECTION AND ALIGNMENT

In order to design primers able of amplifying influenza A, B and RSV circulating viruses, we used NCBI (https://www.ncbi.nlm.nih.gov) and GISAID EpiFlu™ (https://www.gisaid.org) databases to select and download viral genome sequences. A huge number of sequences are collected in these repositories, uploaded and updated daily by worldwide laboratories. Since influenza and RSV viruses are amongst the most polymorphic human-infecting viruses, we decided to perform a specific selection of the viruses' strains to be analyzed to find the most suitable regions to be targeted by our assay.

6.1. Influenza A

H1N1 and H3N2 viruses for our study were selected considering the WHO recommendations on the composition of influenza virus vaccines from 2010 to 2019

(https://www.who.int/influenza/vaccines/virus/recommendations/en/) and those identified as circulating in humans from 2015 to 2019. Moreover, we identified the most clinically relevant subtypes and strains, using CDC (Centers for Disease Control and Prevention) (https://www.cdc.gov/), WHO (World Health Organization) (https://www.who.int/) information and specialized websites (e.g. Influenza Research Database_IRD) (https://www.fludb.org/brc/home.spg?decorator=influenza) as sources.

RNA genome segments of each H1N1 and H3N2 selected virus were downloaded from NCBI and GISAID EpiFlu™ databases and RNAs multiple alignments were performed using DNASTAR MegAlignPro (DNASTAR Inc. version 13.0) and Geneious Prime (version 11.1; Biomatters Ltd, Auckland, New Zealand) softwares.

H1N1 and H3N2 aligned sequences were screened capturing the variability of RNAs polymorphism and removing all the identical sequences. Among all aligned viruses, influenza A/California/7/2009 (H1N1) pdm09-like virus (recommended in vaccines composition from the 2010 to 2017 northern hemisphere seasons) and influenza A/Texas/50/2012 (H3N2)-like virus (recommended in vaccines composition in the 2014-2015 northern hemisphere season) were selected as reference sequences.

6.2. Influenza B

Similarly to influenza A strategy, also for influenza B virus, genome sequences were selected and downloaded from NCBI and GISAID databases and used to perform multiple alignment. Viruses recommended in vaccines composition from 2010 to 2019 by WHO and those mainly circulating in

humans from 2015 to 2019 were included in our study. The variability of the influenza strains was captured selecting only the most representative strains and removing identical sequences from the alignment.

Among all aligned viruses, influenza B/Brisbane/60/2008-like virus (recommended in vaccines composition in the 2017-2018 northern hemisphere influenza season) was selected as reference sequence.

6.3. RSV

RSV genome sequences were download from NCBI database. In particular, we downloaded RSV genome sequences collected in the NCBI BioProject PRJNA262901. The BioProject database provides an organizational framework to access information about research projects with links to data that were or will be deposited into archival databases maintained at members of the International Nucleotide Sequence Database Consortium (INSDC, which comprises the DNA DataBank of Japan (DDBJ), the European Nucleotide Archive at European Molecular Biology Laboratory (ENA) and GenBank at the National Center for Biotechnology Information (NCBI)). PRJNA262901 BioProject chosen was created to increase the understanding of antigenic evolution of RSV, identify key genomic signatures of virulence and disease severity by comparing RSV sequences from patients with mild disease to those from patients with severe disease. For this reason, sequences collected could be considered representative of the genetic heterogeneity of the virus. Downloaded sequences were aligned to choose the most conserved regions of the genome, to be targeted by RT Q-LAMP primer design strategy.

Importantly, aligned sequences were screened capturing the variability of RNAs polymorphism and removing all the identical sequences.

Among all aligned viruses, NC_038235.1 Human orthopneumovirus Subgroup A and NC_001781.1 Human orthopneumovirus Subgroup B were selected as reference sequences.

6.4. IC

The IC is made from MS2 bacteriophage particles. In details, MS2 genome Reference Sequence (NC_001417.2 phage MS2 genome) was taken as target for RT Q-LAMP primers manual design, supported by the use of DiaSorin LAMP design software (LAMPZero).

7. SAMPLES COLLECTION AND PREPARATION

7.1. cDNA plasmids

For the preliminary RT Q-LAMP primer sets screening it was necessary the use of plasmids containing the genome fragment of the influenza and RSV reference sequences and the targeted genome fragment of MS2 bacteriophage.

The size of the insertions for influenza A H1N1, influenza A H3N2, Influenza B, RSV-A, RSV-B and MS2 phage were respectively 1062, 1064, 1224, 1256, 1176 and 1130 bp (Figure 12). Plasmids were produced with Invitrogen GeneArt® Gene Synthesis by Thermo-Fisher Scientific (Waltham, MA, USA). The RNA sequences were synthetized as DNA, cloned into pMA-T vector and transformed into *E. Coli*. The constructs were purified and sequence-verified.

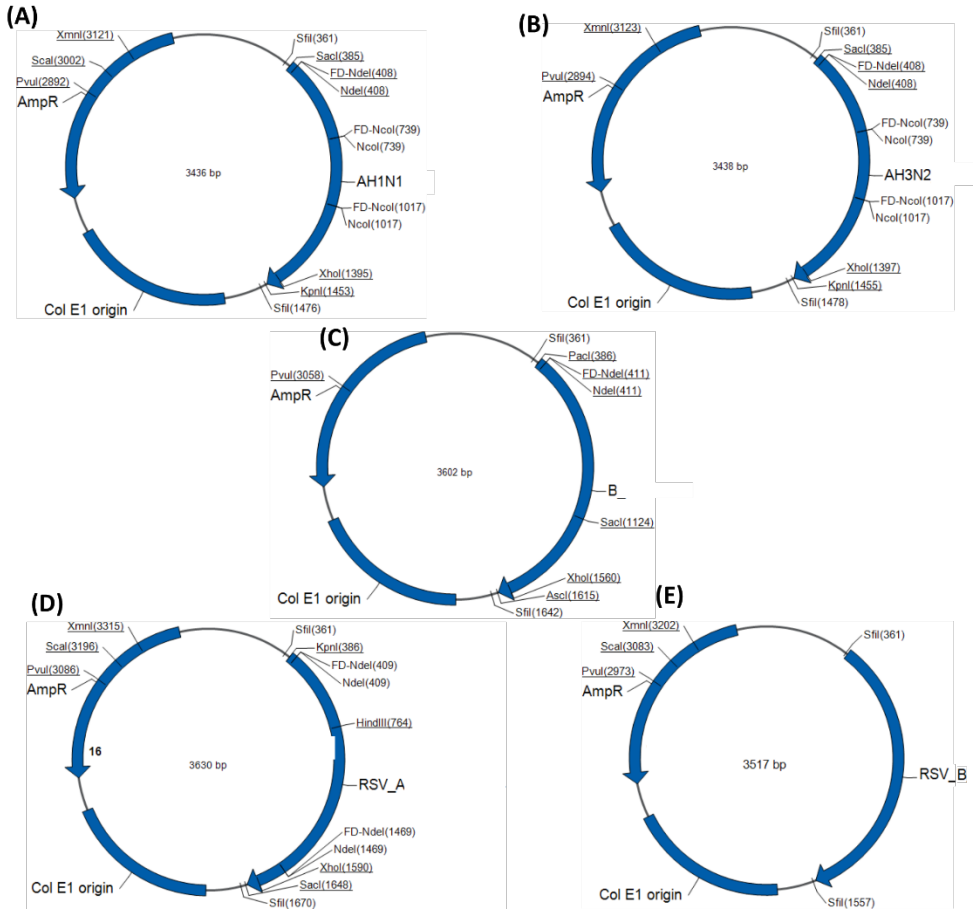


Figure 12. Plasmid's map provided by Thermo-Fisher Scientific for influenza A H1N1 (A), influenza A H3N2 (B), influenza B (C), RSV-A (D) and RSV-B (E).

7.2. SynRNAs

In order to evaluate preliminary performance of the reverse-transcription step of the RT-Q-LAMP, custom synthetic RNAs, harboring the genome target amplicons of the influenza and RSV viruses and the MS2 phage amplicon, were synthetized, purified and sequence-verified by Bio-Synthesis Inc (Lewisville, TX, USA). These oligonucleotides are represented by a fragment

of 498 bp, 451 bp, 415 bp, 456 bp, 456 bp and 560 bp of RNA respectively for influenza A H1N1, influenza A H3N2, influenza B, RSV-A, RSV-B and MS2 phage.

The constructs were generated using bacteriophage polymerase such as T7, T3 or SP6 and purified using RNase free PAGE electrophoresis combined with HPLC, sequence-verified with mass spectrometric analysis and provided with at least 90% final purity. Final RNA transcripts were treated with DNase to degrade DNA. The final products have concentrations confirmed by droplet digital PCR (ddPCR) quantitation.

synRNAs were serially diluted into DEPC-treated water and assayed in duplicates or triplicates with RT Q-LAMP.

7.3. Inactivated virus stocks

To test RT Q-LAMP assay on unprocessed samples, we also used commercially available viruses, produced by Virapur LLC (San Diego, CA, USA). Virus strains were growth in cells, β-propiolactone inactivated, quantified by ddPCR and tested for sterility.

In particular, the following viruses were provided and used:

- A/Michigan/45/2015 (H1N1) pdm09-like virus
- A/Singapore/INFIMH-16-0019/2016 (H3N2)-like virus
- B/Colorado/06/2017-like virus (B/Victoria/2/87 lineage)
- A/California/7/2009 (H1N1) pdm09-like virus
- A/Hong Kong/4801/2014 (H3N2)-like virus
- A/Texas/50/2012 (H3N2)-like virus
- B/Brisbane/60/2008-like virus

- B/Phuket/3073/2013-like virus
- MS2 Bacteriophage
- RSV-A2
- RSV B CH93(18)-18

Available viruses were diluted into Phosphate-buffered saline (PBS) producing artificial samples mimicking patient clinical samples. MS2 bacteriophage, in the final condition, was mixed into the reaction mix, to verify the successful annealing of primers on the encapsulated RNAs.

7.4. Positive and negative clinical specimens

The final evaluation of RT Q-LAMP assay was performed on patients with influenza or RSV diseases. A total of 90 positive nasal and nasopharyngeal swabs (NPS) were collected from Italian and U.S. hospitals from subjects who gave their informed consent. In order to evaluate the specificity of the RT Q-LAMP assay, also 30 influenza A, B or RSV negative NPS or bronchoalveolar lavage (BAL) clinical samples, were tested. Table 3 describes tested clinical specimens.

Clinical Specimens		
N#	**Sample Type**	**Positivity**
10	Nasal/NP swab	Influenza A H1N1
20	Nasal/NP swab	Influenza A H3N2
30	Nasal/NP swab	Influenza B
30	Nasal/NP swab	RSV
22	Nasal/NP swab	None
8	BAL	None

Table 3. Clinical samples collected from Italian and U.S. hospitals tested to validate influenza A, B and RSV quadruplex final assay.

8. LIAISON MDX INSTRUMENT AND DIRECT AMPLIFICATION DISC (DAD)

The Liaison MDX instrument is an innovative and powerful thermocycler specifically developed by DiaSorin for the execution of Real-time PCR assays (Figure 13A). In our set up, the instrument was used to perform RT Q-LAMP assay. It can be used with a particular consumable 8-well disc, named Direct Amplification Disc (DAD) (Figure 13B). Fifty µL of reaction mix is loaded into DAD "reaction" well and 50 µL of primary (directly harvest from patient, not extracted) sample is loaded into "sample" well. Wells are subsequently closed with DAD foil. After loaded the disc into the instrument, the run can start. Specially design reservoirs in the disc "dose" 10 µL of sample volume and 40 µL of reaction mix. A laser opens a valve allowing sample to move to the amplification chamber, thanks to centrifugal forces. Fluid checks system is implemented to ensure sample addition which prevents false negatives if a sample is accidentally not loaded. In the amplification chamber, high temperatures and centrifugation lyse the virus present in the sample allowing the release of the nucleic acid into the media. Then the laser opens a second valve, allowing 40 µL of reagent mix to reach the amplification chamber thanks to centrifugal force. When sample and reagents are in contact, RT Q-LAMP can start.

(A)

(B)

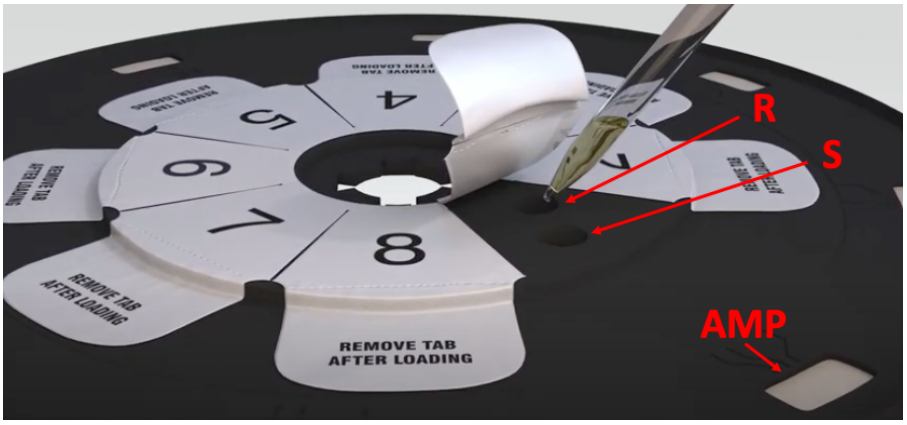


Figure 13. Liaison MDX (A) and DAD (B). DAD is characterized by a "R" and "S" wells, in which the reaction mix and sample are loaded respectively. After valve openings, the centrifugation force allows both sample and mix to reach amplification chamber "AMP", where RT Q-LAMP can start.

Liaison MDX software provides easy to understand results with the ability to check amplification curves both in real-time and after the run. The software also plots QC Charts and can be bi-directionally interfaced with LIS for easy integration into lab workflow.

This instrument is characterized by 4 detection channels:

- Channel 1: excitation 475 nm and detection 520 nm
- Channel 2: excitation 520 nm and detection 560 nm
- Channel 3: excitation 580 nm and detection 610 nm
- Channel 4: excitation 640 nm and detection 682 nm

9. DESIGN AND VALIDATION OF RT Q-LAMP REACTION

9.1. Primer sets design

The primer sets for influenza and RSV were designed targeting genome conserved region of the viruses identified from sequences databases previously obtained (as described in section 6). IC primers were designed on phage MS2 genome NCBI Reference Sequence NC_001417.2.

Primer sets were initially designed both manually and using DiaSorin LAMP design software (LAMPZero), according to the following criteria:

1) Primers length between 17 bp and 25 bp.
2) The Tm for each region is designed to be about 65°C (64 - 66°C) for F1c and B1c, about 60°C (59 - 61°C) for F2, B2, F3 and B3 and about 65°C (64 - 66°C) for the loop primers.
3) GC content between about 40% to 65%.

4) The 3’ ends of F2/B2, F3/B3 and LF/LB and the 5’ end of F1c/B1c are designed so that the free energy is –4 kcal/ mol or less. The 5’ end of F1c after amplification corresponds to the 3’ end of F1, so that stability is important. The change in free energy (ΔG) is the difference between the product free energy and the reactant free energy.
5) Distance from the end of F2 to the end of B2 (the region amplified by the LAMP method) is between 120 bases and 160 bases. The primers are also designed so that the distance from the 5’ end of F2 to the 5’ end of F1 (the portion that forms the loop) is between 40 bases and 60 bases. The primers are also designed so that the distance between F2 and F3 is between 0 to 60 bases.
6) No self-annealing and no secondary structures.

Designed primer sets were analyzed with the software Visual OMP (DNASoftware, Ann Arbor, USA). The characteristic of this software is to provide also predictions of secondary structures, which can help in excluding primer sets that can give primer-dimers amplification. It performs the calculation of Tm by the “nearest neighbor” method, taking also in consideration the different factors influencing the reaction, such as temperature, primers, salts and glycerol concentration. *In silico* selection was based on the absence of primer-dimers and non-specific binding to other portions of the target sequence.

In final sets, one of the primers for each set was substituted with Q-Probe, which is conjugated with a unique fluorophore that is quenched in the presence of the target sequence. The reduction of fluorescence is used to monitor the reaction by a concept called time threshold (Tt), analogous to

PCR threshold cycle (Ct) but measured in minutes (Figure 14). It allows the real time detection of the amplification and the discrimination between targets (viruses and IC).

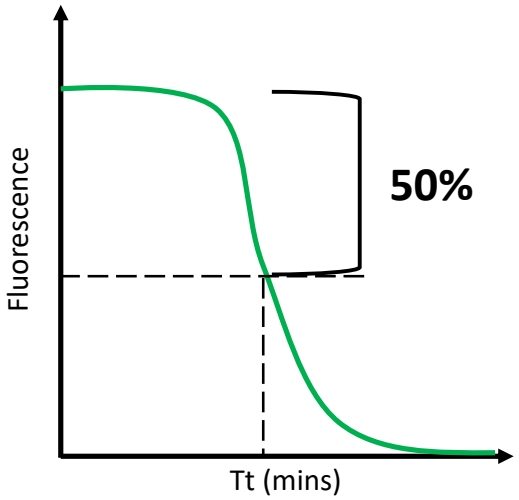


Figure 14. Schematic representation of RT Q-LAMP curve: targets amplification is monitored through the reduction of fluorescence allowed thanks to the use of Q-probe. The annealing of the Q-probe to the target and subsequent isothermal amplification decrease fluorescence. In this application, Threshold time (Tt) represents the real-time LAMP measurement analogous to PCR threshold cycle (Ct).

For the assay, four different customized fluorophores were used, specifically read by the channels of the Liaison MDX instrument. Customized fluorophores were produced by Cyanagen (Bologna, Italy), with different emission wavelength so that they could be read in four different channels of Liaison MDX instrument without crosstalk effect.

For the quadruplex reaction, influenza A was labelled with FAM, influenza B with JOE, RSV with CFR610 and IC with Q670. FAM has an absorption wavelength of 495 nm and emits at 520 nm, JOE has an absorption wavelength of 520 nm and emits at 548 nm, CFR610 has an absorption

wavelength of 590 nm and emits at 610 nm and Q670 has an absorption wavelength of 644 nm and emits at 670 nm. The primers and probes were synthesized by SGS DNA (Köping, Sweden).

9.2. Primer sets selection

The sequence specificity of the primers (i.e. absence of homology with other viruses, bacteria, parasites or human genome sequences) was preliminarily *in silico* checked by use of online NCBI/Nucleotide Blast software (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Experimental primers selection was performed testing each set singularly (in a simplex strategy) at standard conditions on the specific plasmid ($1x10^6$, $1x10^5$, $1x10^4$ copies/mL) plasmids to test the speed of amplification and on Tris-EDTA (TE) Buffer samples (as Non Template Control – NTC) to preliminarily test the specificity.

Importantly, plasmids were preliminary denatured by heating them at 75°C for 5 minutes and immediately chilled on ice to prevent re-naturation.

Primer sets were tested and screened using Q-LAMP standard condition. In this set up each reaction mixture contains the primer set specific for the target (F3/B3 0.2 µM, FIP/BIP 1.6 µM, LF or LB 0.8 µM) and the specific probes 0.8 µM, dNTPs 1.4 mM each, 4 mM $MgSO_4$, Tris-HCl 20 mM, KCl 10 mM, *Bst* DNA polymerase large fragment (New England Biolabs, Ipswich, MA, USA) 8 units per reaction (U/rx) and distilled water (up to the final volume of 40 µL). Thermal protocol used was standard: 65°C for 60 minutes.

The rapidity was determined as the capability to complete the amplification of the $1x10^6$ copies/mL of plasmid within 20 minutes.

The primer sets that passed the selection had at least 90% of specificity (meaning absence of primer-dimers and non-specific signal in NTC) and the amplification of all tested plasmids dilutions should be completed within 60 minutes.

9.3. Influenza A, B and RSV simplex reactions optimization

Influenza A, B and RSV primer sets were initially optimized for their ability to reverse-transcribe viruses RNAs with sufficient sensitivity, in a simplex approach.

To this aim, serial dilutions of synRNAs ($1x10^5$, $1x10^4$ and $1x10^3$ copies/mL, 3 replicates for each dose) into DEPC-treated water were tested changing reaction conditions: temperature, buffer, magnesium and deoynucleotides triphosphate (dNTPs). Specificity was evaluated as absence of primer-dimers signals in NTC samples, represented by DEPC-treated water. Influenza A simplex is mainly used as prototype assay. Best conditions in terms of speed and specificity, obtained with influenza A, were subsequently confirmed with influenza B and RSV simplex assays.

Fast, specific and sensitive best reaction condition was successively tested with unprocessed samples, obtained performing dilutions of inactivated viruses (influenza A and B $1x10^4$ copies/mL, 3 replicates for each virus) into PBS, producing artificial samples mimicking patient clinical specimens. Thermal protocol was changed and optimized to allow a preliminary high temperature and centrifugation step inside DAD allowing the release of the nucleic acid from the virus into the media, followed by a unique isothermal step during which nucleic acids were amplified. Different thermal treatments

were tested looking for the most promising one, providing viral particles amplification Tt as close as possible to the same dose of synRNAs, considering acceptable a delay of 5 minutes maximum.

9.4. Influenza A, B, RSV and IC multiplex reaction optimization

Best influenza A, influenza B, RSV and IC primer sets and simplex reaction conditions were finally tested and validated in a multiplex approach, mixed in the same reaction mix.

Preliminary performance data obtained on plasmids, synRNAs and inactivated viruses were repeated with the multiplex reaction mix, verifying sensitivity and specificity data further optimizing reaction conditions.

To this aim, inactivated viral stocks were diluted ($1x10^4$ copies/mL) into PBS, producing artificial samples mimicking patient clinical specimens and tested in triplicates. For the same target amount, 10 minutes of delay in the amplification, compared with the relative simplex amplification, was considered acceptable.

Importantly, in this reaction optimization process, different conditions were tested, changing reactants amounts (i.e. primers and enzyme amount).

9.5. IC target definition and evaluation

The use of the IC in RT Q-LAMP is an important quality control. An IC for diagnostic assays should be stable, noninfectious and absent from clinical samples. It is very important to control the effectiveness of nucleic acid reverse-transcription and to evaluate the presence of reaction inhibitors

allowing validating negative results. In fact, if the viral genome is amplified in the sample, it means that no inhibitors are present in the sample and that the PCR was successful: in this case the amplification in the IC is not a fundamental parameter to validate the result. Conversely, the amplification of the IC has to occur at least in negative samples, meaning the successful completion of the PCR reaction, validating the results, as described in section 5.

In the final assay set up, β-propiolactone inactivated MS2 bacteriophage is included in the reaction mix. For this reason, tenfold serial dilutions ($1x10^3$, $1x10^2$, 10 copies/reaction) were diluted directly into the quadruplex format reaction mix and seven replicates of NTC samples (PBS) were tested for each MS2 Bacteriophage dose so as to choose the amount to be included in the reaction mix giving 100% amplification around 30 Tt.

10. INFLUENZA A, B AND RSV QUADRUPLEX ASSAY ANALYTICAL PERFORMANCE

10.1. Analytical sensitivity and specificity

Analytical sensitivity is determined by the Limit of Detection (LoD) value, which represents the lowest amount of target that can be detected in at least 95% of replicates. It was evaluated performing serial dilutions of A/Michigan/45/2015 (H1N1) pdm09-like virus, A/Singapore/INFIMH-16-0019/2016 (H3N2)-like virus, B/Phuket/3073/2013-like virus and RSV-A2 inactivated virus stock in PBS. For each virus, four different doses ($1X10^5$, $1X10^4$, $1X10^3$, $1X10^2$ copies/mL) were tested in forty replicates.

Analytical specificity was assessed on 100 replicates, represented by both DEPC-treated water and PBS. In details, 50 replicates of DEPC-treated water and 50 replicates of PBS were tested. All results must be validated through the correct amplification of the IC in 682 nm channel that excludes the presence of inhibitors and ensures the use of correct reaction conditions.

10.2.Analytical reactivity

We verified that influenza A, B and RSV RT Q-LAMP was able to detect additional influenza A, influenza B and RSV strains not preliminarily evaluated during reaction optimization steps.

Test samples were prepared diluting different influenza A, B or RSV strains from stocks (Table 4) that were quantified and titered in TCID50/mL, CEID50/mL, EID50/mL or other industry acceptable units. Test samples were contrived in PBS and tested in triplicates. To validate the results, in each run, one replicate of $1x10^4$ copies/mL of influenza A, B and RSV inactivated viruses (reference strains) diluted in PBS, mixed together and one NTC (PBS) were included. Analytical reactivity should be observed with all of the organisms tested.

Organisms		
Influenza A viruses	**Influenza B viruses**	**RSV**
A/California/4/2009 (H1N1)	B/Brisbane/33/2008	ATCC-2012-10
A/California/12/2012 (H1N1)	B/Brisbane/60/2008	A 1997/12-35
A/Massachusetts/15/2013 (H1N1)	B/Florida/02/2006	A 1998/12-21
A/Mexico/4108/2009 (H1N1)	B/Lee/40	A 1998/3-2
A/New York/18/2009 (H1N1)	B/Nevada/03/2011	A 2000/3-4
A/Brisbane/59/07 (H1N1)	B/Texas/02/2013	A 2001/2-20
A/Hawaii/15/2001 (H1N1)	B/Victoria/304/2006	A 2001/3-12
A/New Caledonia/20/99 (H1N1)	B/Christchurch/33/2004	A Long
A/Solomon Island/03/06 (H1N1)	B/Florida/07/04	B 9320
A/Taiwan/42/06 (H1N1)	B/Florida/04/2006	B/Wash/18537/62
A/WS/33 (H1N1)	B/Guangdong-Liwan/1133/2014	B/WV/14617/85
A/Brisbane/10/07 (H3N2)	B/Maryland/1/59	
A/California/02/2014 (H3N2)	B/Great Lakes/1739/54	
A/New York/55/2004 (H3N2)	B/Panama/45/90	
A/Ohio/02/2012 (H3N2)	B/Phuket/3073/2013	
A/Port Chalmers/1/73 (H3N2)	B/Utah/9/2014	
A/Rhode Island/01/2010 (H3N2)	B/Wisconsin/01/2010	
A/Santiago/7981/2006 (H3N2)	B/Allen/45	
A/Switzerland/9715293/2013 (H3N2)	B/Hong Kong/5/72	
A/Texas/50/2012 (H3N2)	B/Taiwan/2/62	
A/Wisconsin/67/05 (H3N2)	B/Colorado/06/2017	
A/Indiana/08/2011 (H3N2)	B/Michigan/09/2011	
A/Minnesota/11/2010 (H3N2)	B/Texas/81/2016	
A/Hong Kong/33982/2009(H9N2)-PR8-IDCDC_RG26	B/Washington/02/2019	
A/Singapore/INFIMH-16-0019/2016 (H3N2)	B/New Hampshire/01/2016	
A/Michigan/45/2015 (H1N1)		
A/Minnesota/19/2011 (H1N2)		
A/Hong Kong/4801/2014 (H3N2)		
A/Kansas/14/2017 (H3N2)		
A/Brisbane/02/2018 (H1N1)		
A/Christchurch/16/2010 (H1N1)		
A/Hong Kong/2671/2019 (H3N2)		
A/Guandong-Maonan/1536/2019 (H1N1)		
A/Perth/16/2009 (H3N2)		
A/NY/02/09 8H1N1)		

(1) TCID50/mL = Tissue Culture Infectious Dose
CEID50/mL = Chicken Embryo Infectious Dose
EID50/mL = Egg Infectious Dose
PFU/mL = Plaque forming units

Table 4. *In vitro* analytical reactivity of RT Q-LAMP influenza A, B and RSV quadruplex assay. Thirty-five influenza A, 25 influenza B and 11 RSV viruses quantified and titered, were tested in triplicates.

10.3.Cross-reactivity

Cross-reactivity of the assay was evaluated by testing the ability of the assay to exclusively identify influenza A and/or influenza B and/or RSV viruses, with no cross-reactivity to other organisms that are closely related or cause similar symptoms or are present as normal flora in NPS specimens. Test samples were prepared diluting different bacteria, fungi, parasites and/or viruses from stocks that were quantified and titered in CFU/mL, IFU/mL, TCID50/mL, copies/mL, or other industry acceptable units (Table 5). Test samples were contrived in PBS and assayed in triplicates. To validate the results, in each run, one replicate of $1x10^4$ copies/mL of influenza A, B and RSV inactivated viruses (reference strains) diluted in PBS, mixed together and one NTC (PBS) were included. Cross-reactivity should not be observed with any of the organisms tested.

Influenza A, B and RSV Cross-reactivity	
Organism	**Tested Concentration (1)**
Adenovirus Type 1	1x10^6 TCID50/mL
Adenovirus Type 7A	1x10^5 TCID50/mL
Bordetella pertussis A639	1x10^7 CFU/mL
Chlamydia pneumoniae	1x10^6 IFU/ml
CMV 169	1x10^5 TCID50/mL
Coronavirus 229E	1x10^5 TCID50/mL
Coronavirus OC43	1x10^5 TCID50/mL
Coronavirus NL63	1x10^5 TCID50/mL
Enterovirus Type 71	1x10^5 TCID50/mL
Epstein Barr Virus	1x10^6 copies/mL
Escherichia coli	1x10^7 CFU/mL
Haemophilus influenzae	1x10^7 CFU/mL
Metapneumovirus 9	1x10^6 TCID50/mL
Mycobacterium tuberculosis (genomic DNA)	1x10^7 copies/mL
Mycoplasma pneumoniae	1x10^6 CCU/mL
Neisseria meningitidis	1x10^6 CFU/mL
Parainfluenza 1	1x10^5 TCID50/mL
Parainfluenza 2	1x10^6 TCID50/mL
Parainfluenza 3	1x10^5 TCID50/mL
Pseudomonas aeruginosa	1x10^7 CFU/mL
Rhinovirus 1A	1x10^5 TCID50/mL
SARS-CoV2	1x10^6 TCID50/mL
Staphylococcus aureus, COL	1x10^7 CFU/mL
Streptococcus pneumoniae	1x10^7 CFU/mL
Streptococcus pyogenes, M1	1x10^7 CFU/mL
Streptococcus salivarius	1x10^7 CFU/mL

(1) TCID50/mL = Tissue Culture Infectious Dose
CFU/mL = Colony Forming Units
IFU/mL = Infectious Units

Table 5. Cross-reactants tested with influenza A, B and RSV RT Q-LAMP quadruplex assay. Reported bacteria, viruses and parasites were tested with RT Q-LAMP quadruplex assay to verify absence of cross-reactivity.

11. INFLUENZA A, B AND RSV FINAL ASSAY VALIDATION ON CLINICAL SAMPLES

To validate our final reaction condition, we tested 120 nasopharyngeal clinical samples collected from Italian and U.S. hospitals from subjects who gave their informed consent. Each sample was preliminary tested with

commercially available RT-PCR kit, routinely used in the hospital laboratories. Ninety clinical samples were diagnosed for influenza A, influenza B or RSV infection. In addition, 30 influenza A, B or RSV negative NPS or BAL clinical samples, were tested.

By the combined results of the two assays, we expected four possible results:

A. Samples in which both assays detected no signals, suggesting absence of virus infection (True Negative, TN).
B. Samples in which both assays detected considerable virus signals, indicating presence of viruses and thus suggesting an infection (True Positive, TP).
C. Samples in which RT-PCR assay detected signals, resulted negative by RT Q-LAMP assay. In this case, RT Q-LAMP analysis must be repeated to confirm or deny the result (False Negative, FN).
D. Samples in which RT Q-LAMP assay detected signals resulted negative by RT-PCR assay. In this case, RT Q-LAMP analysis must be repeated to confirm or deny the result (False Positive, FP).

According to these possibilities, the clinical agreement of our assay was calculated through the clinical sensitivity and specificity and through the Positive Predictive Value (PPV) and Negative Predictive Value (NPV):

$$\textbf{Sensitivity \% = TP/(TP+FN)*100}$$

$$\textbf{Specificity \% = TN/(TN+FP)*100}$$

$$\textbf{PPV \% = TP/(TP+FP)*100}$$

$$\textbf{NPV \% = TN/(TN+FN)*100}$$

RESULTS

12. INFLUENZA A, B AND RSV SEQUENCES SELECTION AND ALIGNMENT

For the primer design, we selected and downloaded many different influenza A, B and RSV genome sequences from NCBI and GISAID EpiFlu™ databases. During the sequences selection process, we maintained and/or included the sequences of viruses contained in the annual vaccines from 2010 to 2019 and the most clinically relevant subtypes and strains, identified using CDC (Centers for Disease Control and Prevention), WHO (World Health Organization) and specialized websites (e.g. Influenza Research Database_IRD) as information sources. Importantly, since there are 131 different influenza A virus subtypes, thanks to this selection process we decided to mainly target the H1N1 and H3N2 subtypes, which are the most clinically relevant ones, included in seasonal vaccines. Finally, we excluded viral strains with identical sequence to others already included in our database. This process was particularly crucial because the creation of a good database is fundamental to obtain an assay that effectively responds to clinical needs. Graphical representation of this process is reported in Figure 15.

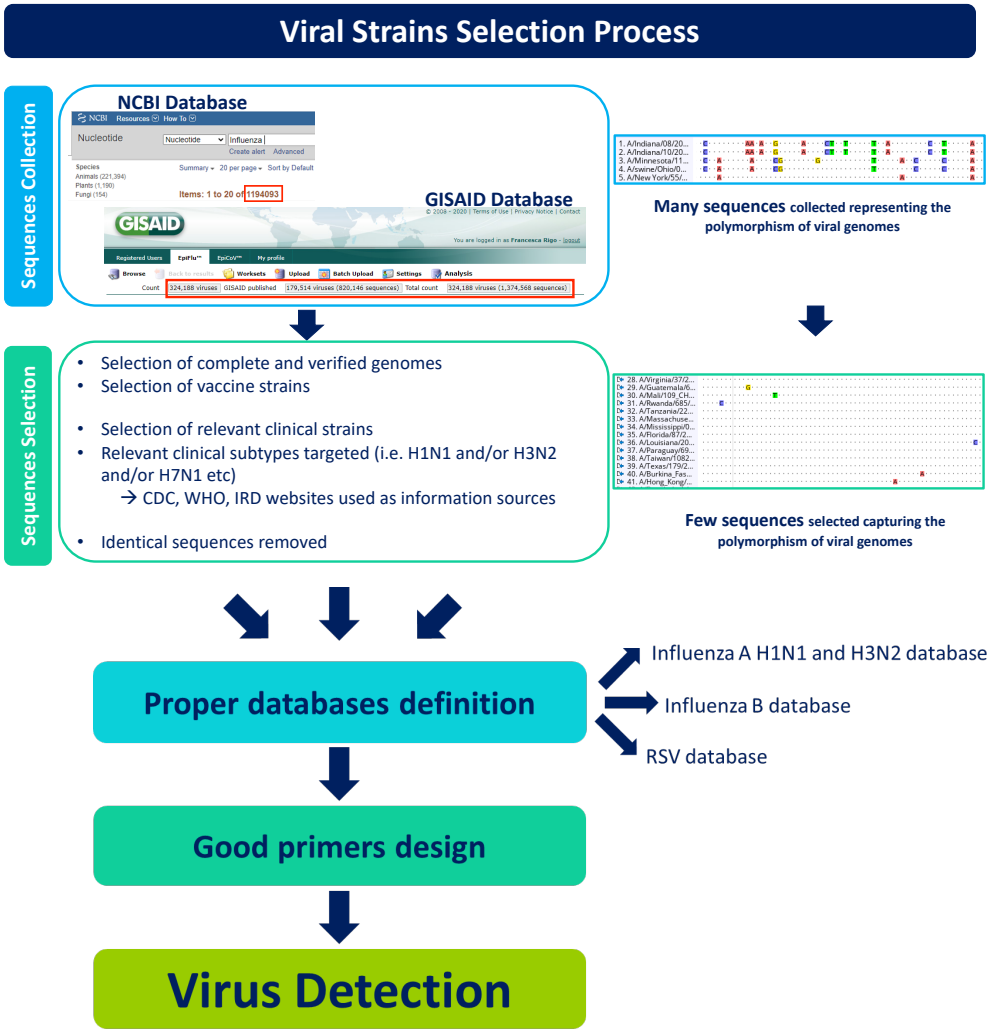


Figure 15. Graphical representation of the viral strains selection process workflow. A huge number of viral genome sequences were collected in NCBI and GISAID EpiFlu™ databases. For this reason, we selected and downloaded only complete and verified genome sequences. Importantly, we included in our study all vaccine strains sequences from 2010 to 2019. Using WHO, CDC and IRD websites as information sources, we selected only the most representative sequences, capturing viral genome polymorphisms. Finally, we analyzed all the sequences collected in our influenza A, B and RSV resulting databases and we decided to retain single sequences representing entire groups of identical sequences. Thanks to this procedure, we succeeded in obtaining three resulting databases (influenza A, B and RSV respectively) collecting the most informative viral genome sequences. This process is particularly important because allowed us to obtain informative

databases of viral genome sequences that were subsequently aligned and used to identify homologous regions to be targeted by primers design strategies.

Thanks to this selection process, a total of 175 influenza A H1N1, 74 H3N2, 216 influenza B, 260 RSV-A and 140 RSV-B viruses were selected for our study, downloaded from NCBI and GISAID databases and aligned using DNASTAR MegAlignPro and Geneious Prime softwares. Selection of the downloaded sequences was performed choosing only the most representative sequences of the variability of the virus (capturing viral genome polymorphisms) and retaining single sequences, representing entire groups of identical sequences. Our final databases resulted in 15 influenza A H1N1, 10 H3N2, 15 Influenza B, 151 RSV-A and 89 RSV-B virus sequences, as schematically reported in Figure 16.

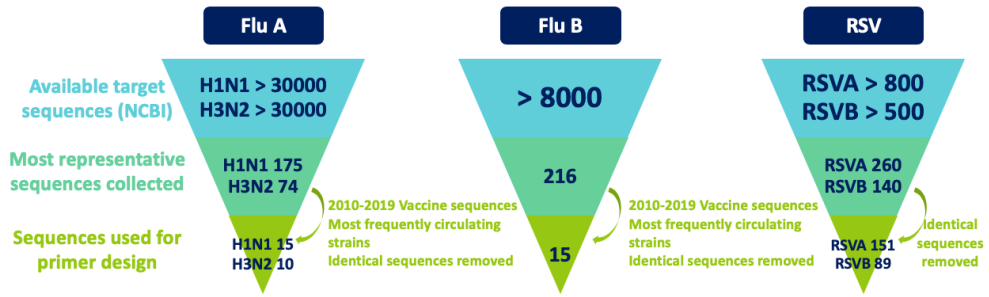


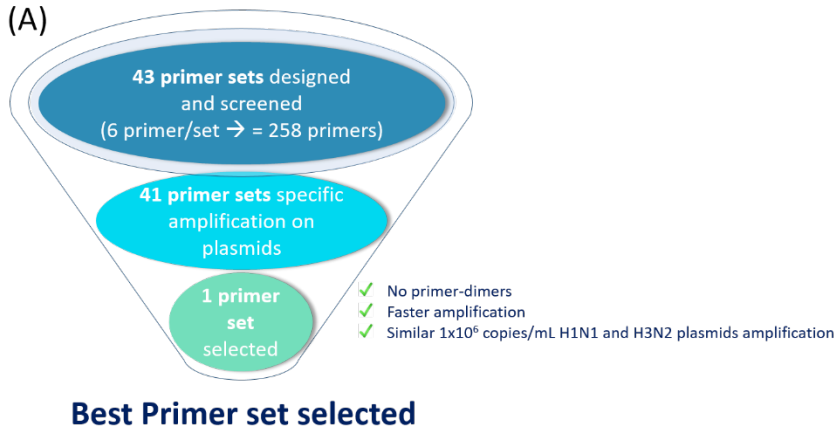
Figure 16. Schematic representation of the virus genome sequences selection result.

13. DESIGN AND VALIDATION OF RT Q-LAMP REACTION

13.1. Influenza A primers design and selection

For influenza A, a total of 43 LAMP primer sets (accounting a total of 258 different primers) were designed. Each primer set included a specific probe labeled with FAM fluorophore. Designed primer sets were tested on plasmids

containing the genome sequence fragments of the influenza A H1N1 or H3N2 sequences (A/California/7/2009 (H1N1) pdm09-like virus, A/Texas/50/2012 (H3N2)). During our preliminary experiments, we observed that $1x10^6$ copies/mL of plasmid for both targets could be quickly amplified, even before 30 minutes. Therefore, it was chosen as main selection criteria for primer sets screening. In details, 41 out of 43 primer sets gave a successful amplification on these targets but only 1 out of 41 set was selected as fastest and most specific (Figure 17A), giving a similar H1N1 and H3N2 amplification, as shown by the amplification curves of $1x10^6$ copies/mL of plasmids reported in Figure 17B.



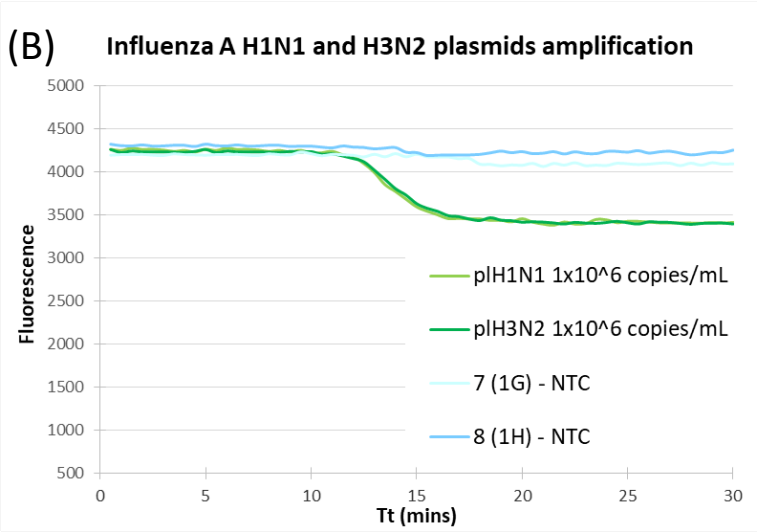


Figure 17. (A) Influenza A primer sets screening process. A total of 43 primer sets targeting influenza A genome were designed. Forty-one out of 43 primer sets gave a successful amplification on plasmids containing the genome fragments of the influenza A H1N1 or H3N2 reference sequences but only 1 out of 41 set was selected as fastest and most specific, giving a similar H1N1 and H3N3 amplification. (B) Q-LAMP amplification curve of influenza A plasmids: final selected primer set allowed simultaneous amplification of both H1N1 and H3N2 1x10^6 copies/mL of plasmids without any non-specific amplification in NTC (TE buffer) samples.

13.2.Influenza B primers design and selection

Several influenza B RT Q-LAMP primers were manually designed (54 primers) on the multiple sequence alignments of influenza B. These primers were combined to obtain nine sets. In this case, the primer set included a specific probe labeled with JOE fluorophore. Similarly to influenza A, the designed primer sets were tested by amplifying a plasmid containing the genome sequence fragment of the influenza reference sequence. Five out of 9 primer sets gave a successful amplification on this target but only 1 out of 5 was selected according to speed and specificity parameters as shown in Figure 18.

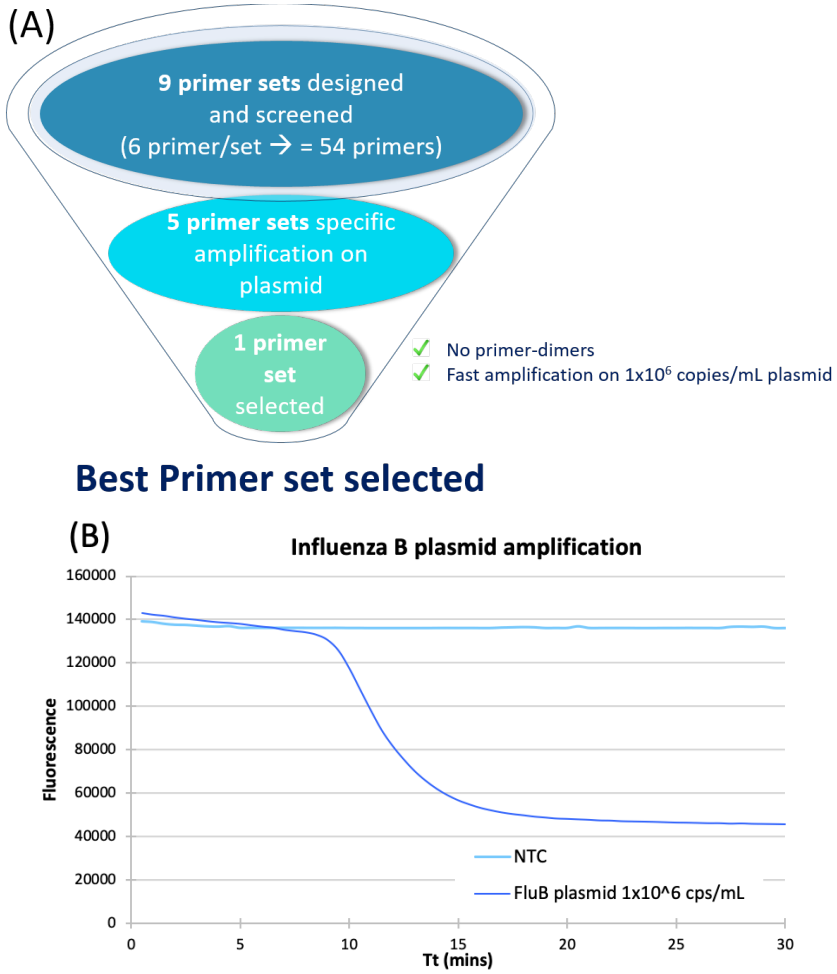


Figure 18. (A) For influenza B, a total of nine LAMP primer sets (accounting a total of 54 different primers) were designed and screened. In this case, primer set included a specific probe labeled with JOE fluorophore. Designed primer sets were tested amplifying 1x10^6 copies/mL plasmid containing the genome fragment of the influenza B reference sequence. In details, 5 out of 9 primer sets gave a successful amplification on plasmid but only 1 out of 5 set was selected as fastest and most specific. (B) Q-LAMP amplification curve of influenza B plasmid using selected primer set. This primer set amplified 1x10^6 copies/mL of plasmid about 15 minutes after the start of the reaction without any non-specific signal in NTC (TE buffer) controls, as shown by the amplification curve reported.

13.3.RSV primers design and selection

In Figure 19 it is reported the RSV primer sets screening process: a total of 13 primer sets (accounting for a total of 78 different primers) were designed. Each primer set included a specific probe labeled with CFR610 fluorophore. Among these 13 primer sets, nine gave a successful amplification on plasmids containing the genome sequence fragments of the RSV-A or RSV-B reference sequences and only 1 out of 9 set was selected as fastest and giving a similar RSV-A and RSV-B amplification of $1x10^6$ copies/mL of plasmids without non-specific signals detected in NTC (TE Buffer) samples (Figure 19B).

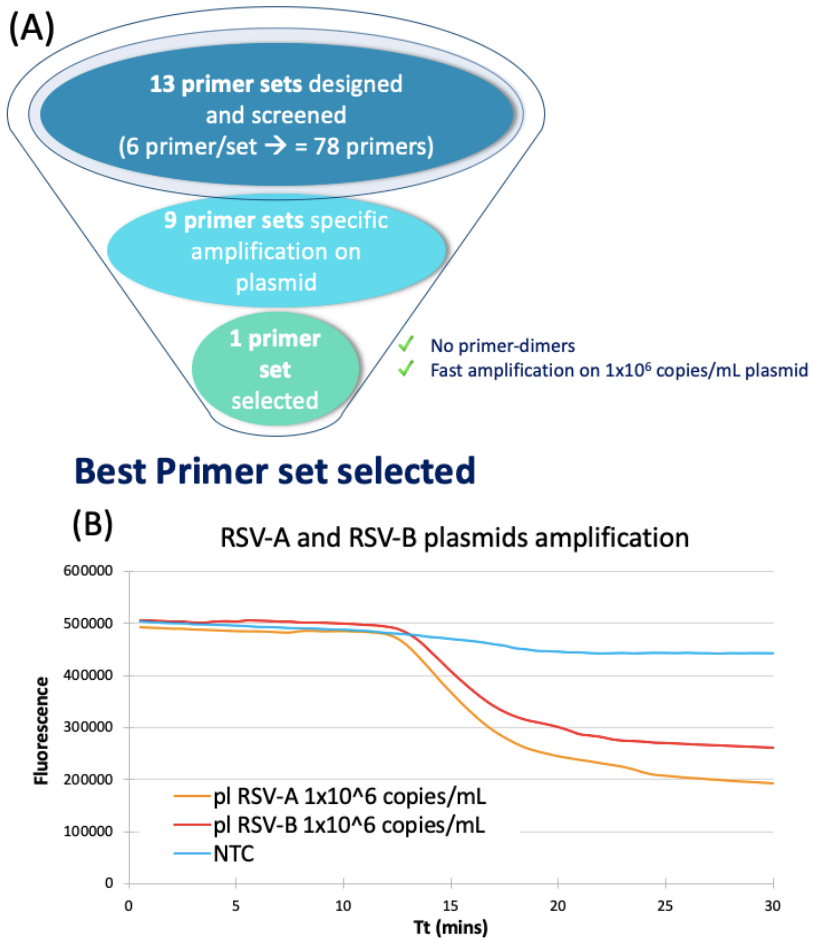


Figure 19. (A) RSV primer sets screening process. A total of 13 primer sets (accounting a total of 78 different primers) were designed. Each primer set included a specific probe labeled with CFR610 fluorophore. All these primer sets were tested amplifying plasmids containing the genome sequence fragments of the RSV-A or RSV-B reference sequences and only 9 out of 13 primer sets gave a successful amplification on these targets. One out of 9 set was selected as fastest and most specific, giving a similar RSV-A and RSV-B amplification of $1x10^6$ copies/mL of plasmids. (B) Q-LAMP amplification curve of RSV-A and RSV-B plasmids: final selected primer set allowed simultaneous amplification of both RSV-A and RSV-B $1x10^6$ copies/mL plasmids without any non-specific amplification in NTC (TE Buffer) samples.

13.4.IC primes design and selection

The use of the IC in RT Q-LAMP is very important to control the effectiveness of nucleic acid reverse-transcription and amplification and to control the presence of inhibitors, consequently validating negative results. MS2 bacteriophage was selected as IC as it is a RNA target so it can be used to verify the reverse-transcription and amplification. MS2 reference sequence was took as target for RT Q-LAMP primers manual design. Eighteen designed primers were combined to obtain three sets. Each primer set included a specific probe labeled with Q670 fluorophore. After preliminary screening tests, we observed that 2 out of 3 primer sets positively amplified a plasmid containing the genome sequence fragment of the MS2 bacteriophage and 1 out of 2 primer set was selected as the most specific (Figure 20). Importantly, in the IC primers selection, the speed of amplification was not a critical feature. The requirement was to have IC amplification later than viral targets in order to not compete with targets amplification for reagents availability.

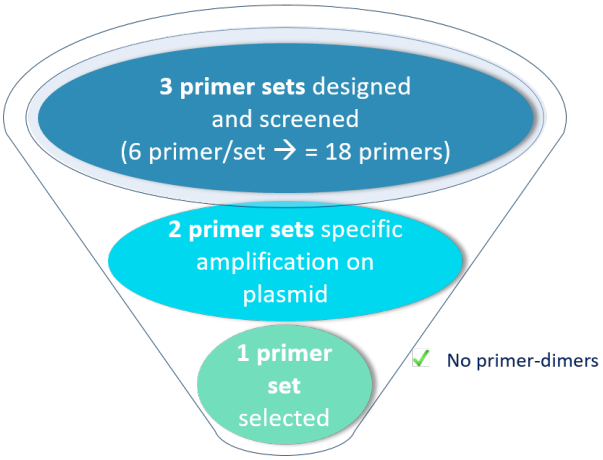


Figure 20. IC Primer sets screening process. The IC was made from MS2 bacteriophage. MS2 Reference Sequence (NC_001417.2 phage MS2 genome) was taken as target for RT Q-LAMP primers set manual design (18 primers combined in 3 sets). Each primer set included a specific probe labeled with Q670 fluorophore. Two out of 3 primer sets positively amplified plasmid containing the genome sequence fragment of the MS2 bacteriophage and 1 out of 2 primer set was selected as the most specific, without primer-dimers signals detected in NTC samples.

13.5.Influenza A, B and RSV reactions optimization

Once the primer sets for all the targets were selected, a deep optimization work needed to be started in order to obtain the best results in terms of sensitivity and specificity.

Many parameters were changed singularly and in combination each other. Each reagent involved in the reaction, in fact, strongly interplays with the others (Figure 21): the change of a single parameter could lead to an improvement in the reaction performance only with a parallel change of other parameters interacting with it.

As a result, these interactions lead to an extremely high number of reaction conditions that can potentially be tested. An example of how this interaction between reagents can guide the optimization process is graphically represented in the Figure 22 and in Figure 23.

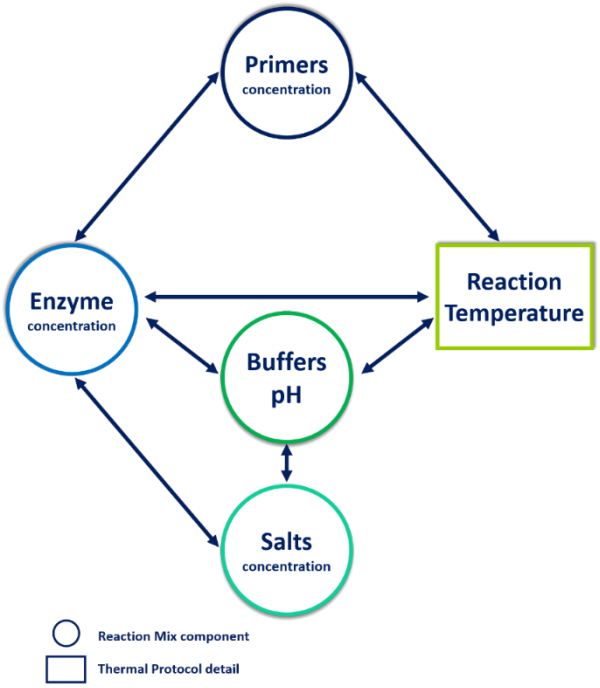


Figure 21. Reagents and parameters involved in the RT Q-LAMP reaction performance. Each reagent in the reaction mix (represented by circled elements) interacts with each others. In parallel, reagents interaction is influenced by temperature conditions (represented by squared elements). For example, enzyme functionality strongly depends from its concentration in the reaction and salts properly support enzyme functionality only if they are added in the right proportion with the enzyme in the reaction. To further increase the complexity, buffer pH influences both enzyme functionality and salts concentration effect on it. Therefore, many different combinations of reagents need to be tested to find the best condition.

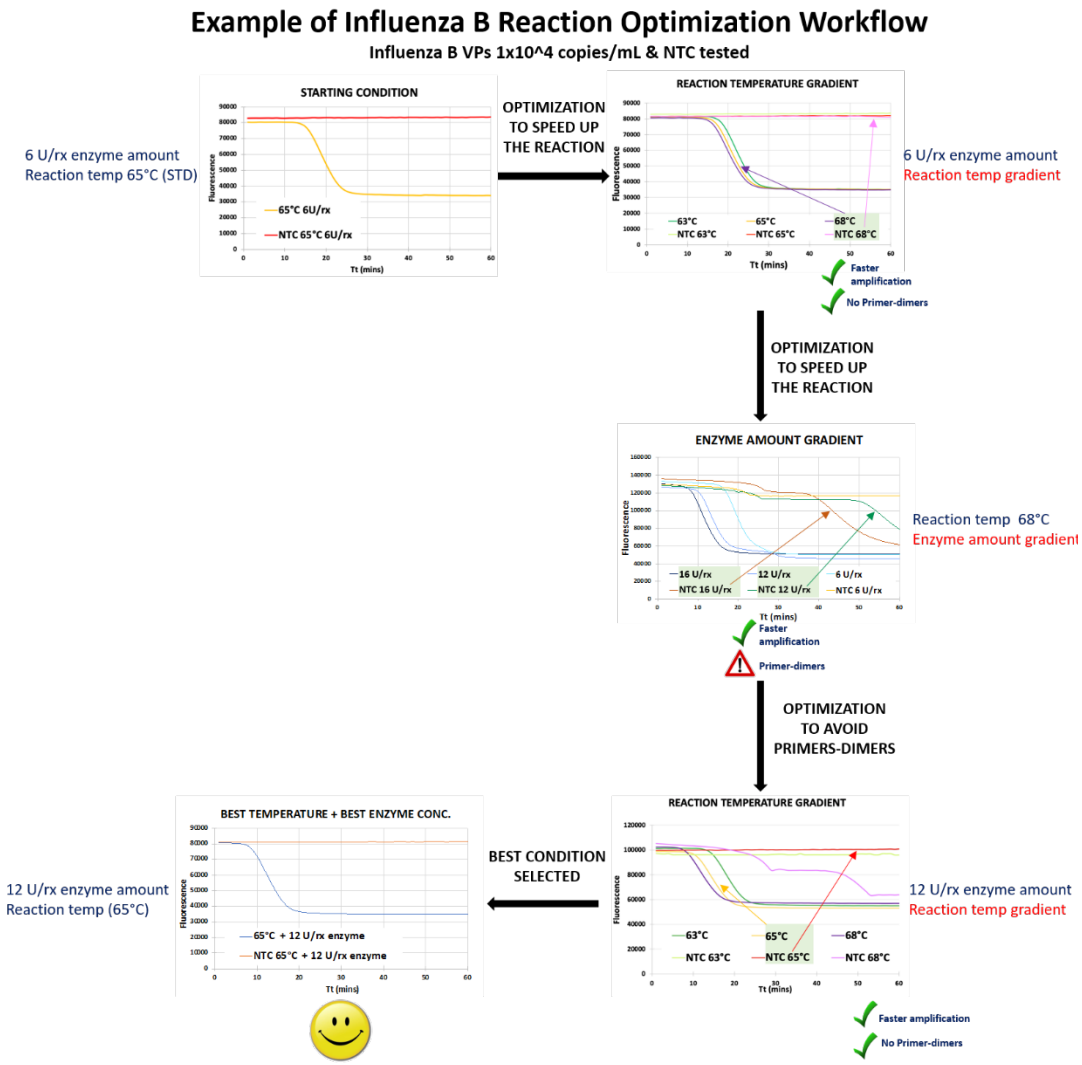


Figure 22. Example of influenza B reaction optimization workflow: starting from a standard condition, characterized by the use of 6 U/rx enzyme and a temperature of 65°C, we optimized the reaction. Firstly, we performed a reaction temperature gradient and we selected 68°C as best in terms of speed and specificity. We subsequently tried to speed up the reaction testing different enzyme concentrations: we observed that both 16 U/rx and 12 U/rx enzyme conditions were promising in terms of amplification speed but caused primer-dimers signals in NTC. For this reason, we changed again the reaction temperature: we observed that the combination of 12 U/rx enzyme at 65°C reaction temperature guaranteed a faster amplification of the target compared to the starting condition, without giving primer-dimers signals in NTC samples.

Example of Influenza A Reaction Optimization Workflow

Influenza A VPs 1x10^4 copies/mL & NTC tested

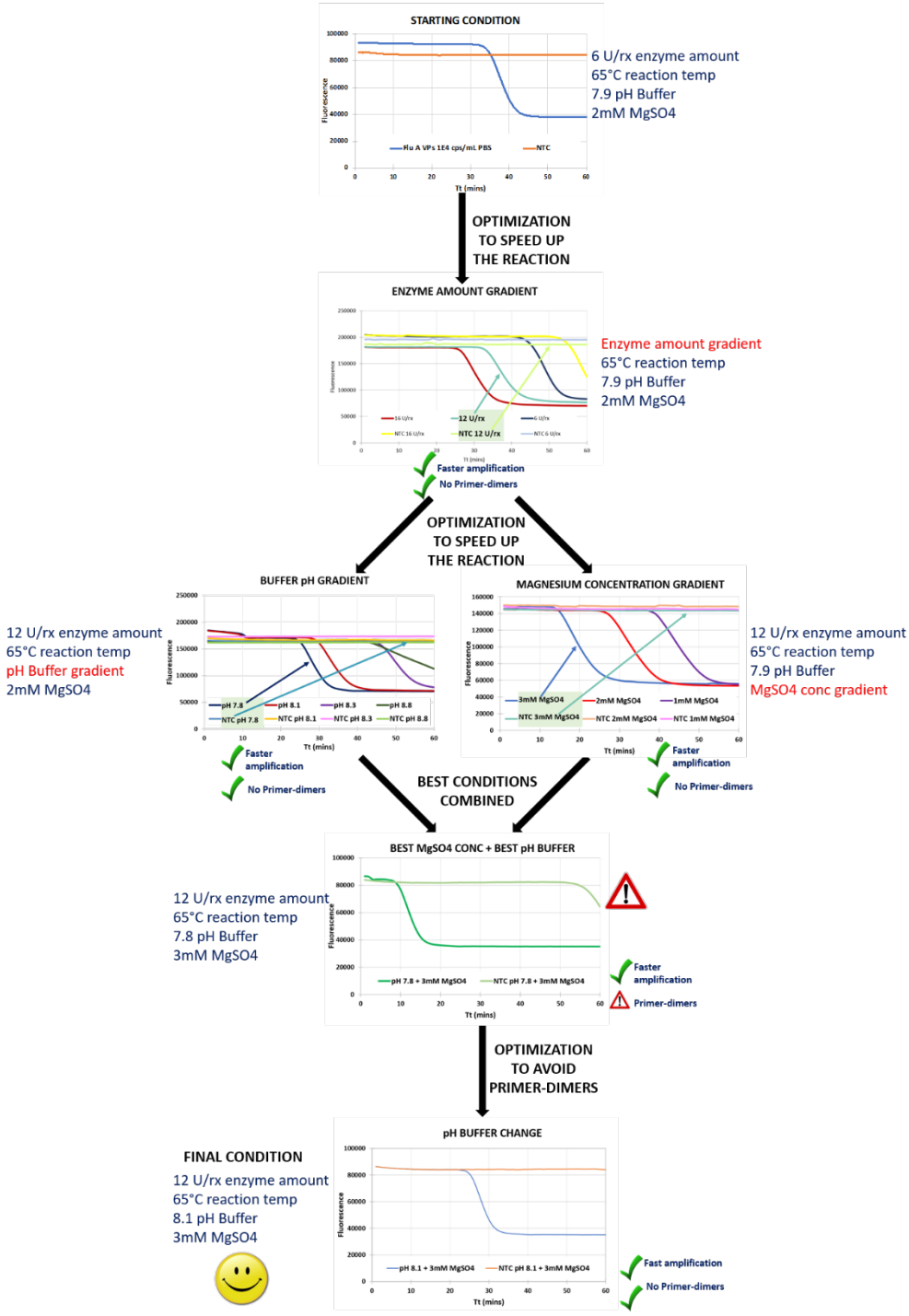


Figure 23. Example of influenza A reaction optimization workflow. We started from a standard condition, characterized by the use of 6 U/rx enzyme, a temperature of 65°C, buffer pH 7.9 and 2mM MgSO4 and we optimized the reaction so as to speed it up. Firstly, we performed an enzyme concentration gradient and we selected 12 U/rx as best in terms of speed and specificity. We subsequently tried to speed up the reaction testing in parallel a buffer pH gradient and a MgSO4 gradient: we observed that 7.8 pH buffer and 3mM MgSO4 conditions were promising in terms of amplification speed and specificity. Therefore, we combined these two best conditions and we observed a fast targets amplification but also primer-dimers signals in NTC. For this reason, we changed again the buffer pH: we observed that the combination of 3mM MgSO4 and buffer pH 8.1 guaranteed a faster amplification of the target compared to the starting condition, without giving primer-dimers signals in NTC samples.

13.5.1.Influenza A, B and RSV simplex reactions optimization

One of the key features of the enzyme selected for the RT Q-LAMP influenza A, B and RSV assay is its ability to both reverse-transcribe and amplify RNA. It is also characterized by a strong strand displacement activity, allowing the reaction to be completed while maintaining isothermal conditions. Before the optimization process, since synRNA needs both to be reverse-transcribed and amplified, it was detected later than the plasmid (which exclusively amplified). Moreover, we observed that unprocessed viral particles were characterized by a slower detection than synRNA or were not even amplified in some cases. Following the optimization process, mainly carried out using the influenza A simplex as prototype assay, we changed many different reaction parameters such us enzyme, primers and salts concentrations, buffer pH and reaction temperature. This process led us to test 198 influenza A reaction conditions. Finally, we managed to obtain both viral particles and synRNAs reverse-transcription and amplification in a single

step, without any preliminary nucleic acid extraction, with results comparable to plasmid in terms of speed (Figure 24).

Similarly to what was done for influenza A, also for the influenza B and RSV simplex assays we modified many parameters, testing a total of 179 and 52 reaction conditions, respectively.

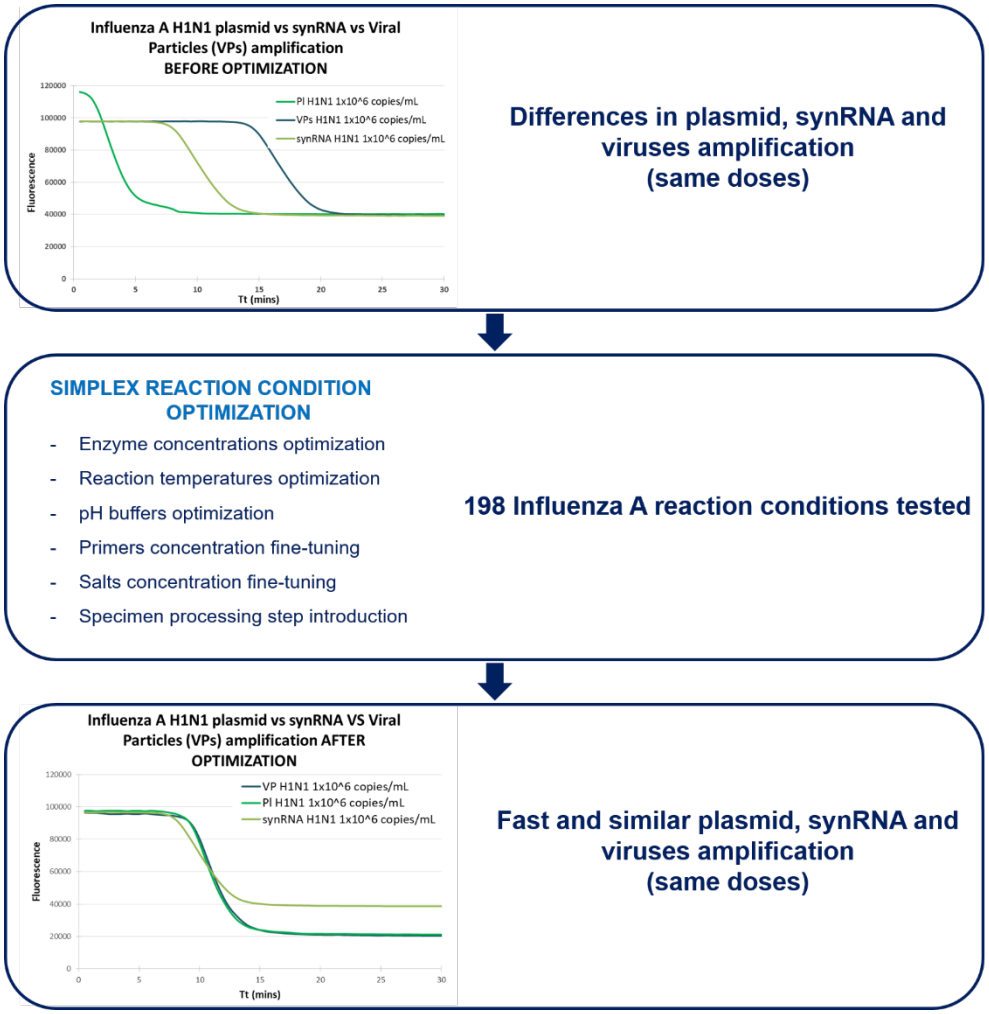


Figure 24. Summary graphical representation of the optimization process of the RT Q-LAMP. Before the optimization, synRNA was detected later than the plasmid. Moreover, we observed that unprocessed viral particles were characterized by a slower detection than synRNA or were not even amplified in some cases. Following the optimization process, mainly carried out using the influenza A simplex as

prototype assay, we managed to obtain both viral particles and synRNAs reverse-transcription and amplification in a single step, without any preliminary nucleic acid extraction, with results comparable to plasmid in terms of speed. During this process, 198 influenza A reaction conditions were tested and analyzed.

13.5.1.1.Reaction Temperature

The enzyme selected for the RT Q-LAMP influenza A, B and RSV assay is characterized by a strong strand displacement activity with an optimum reaction temperature between 60°C and 65°C. A preliminary evaluation of the optimal reaction temperature was performed testing serial dilutions of synRNAs H1N1 ($1x10^5$ – $1x10^4$ – $1x10^3$ copies/mL in triplicates) at different reaction temperatures. According to the data obtained, 65°C reaction temperature was selected as best due to faster amplification compared to other conditions (Figure 25).

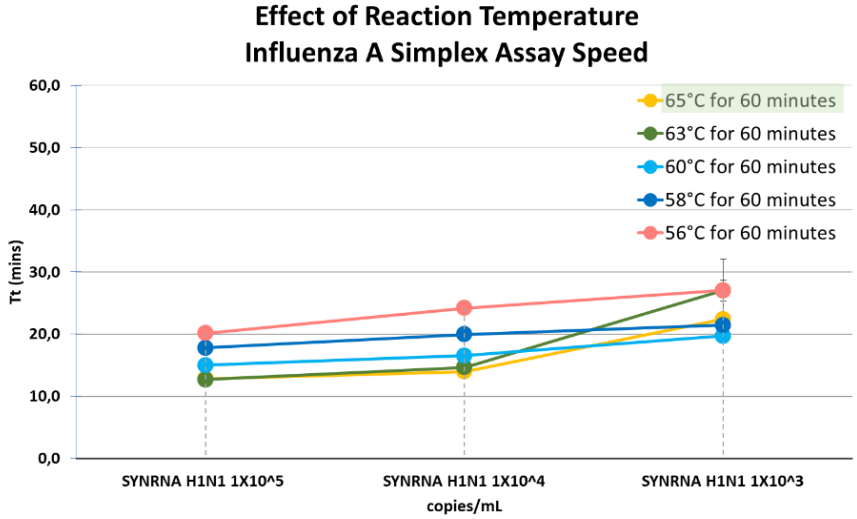
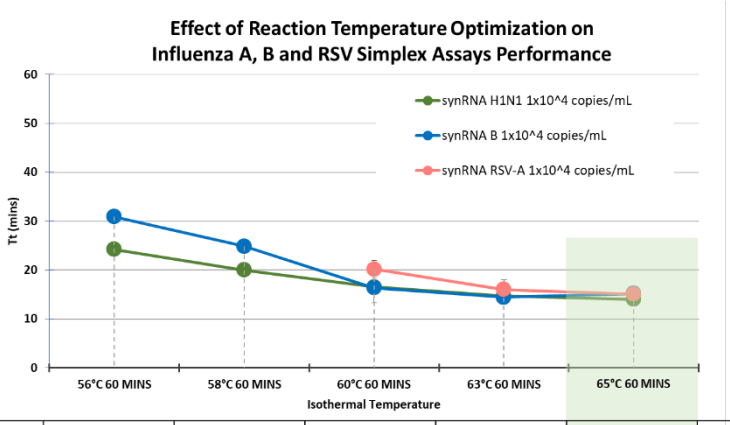


Figure 25. Preliminary influenza A RT Q-LAMP simplex reactions optimization at different temperatures ranging from 56°C to 65°C. A preliminary evaluation of the optimal reaction temperature was performed testing serial dilutions of synRNAs H1N1 ($1x10^5$ – $1x10^4$- $1x10^3$ copies/mL) at different reaction temperatures (56°C – 58°C – 60°C – 63°C – 65°C); the average (AVG) and standard deviation (SD –

represented as bars) of the Threshold Times (Tt) recorded in 500 nm channel were reported.

After preliminary evaluation on influenza A RT Q-LAMP simplex assay, reaction temperature evaluation was performed also with influenza B and RSV simplex assays. In particular, RSV assay was evaluated only between 60°C and 65°C. According to the results obtained with all three simplex assays, 65°C reaction temperature was selected as best for isothermal amplification due to its amplification speed and specificity (Figure 26).



Influenza A	**0%**	**0%**	**0%**	**0%**	**0%**	**Primer-dimers %**
Influenza B	**100%**	**0%**	**0%**	**100%**	**0%**	
RSV	**0%**	**0%**	**0%**	**0%**	**0%**	

Figure 26. Influenza A, B and RSV RT Q-LAMP simplex reactions optimization at different temperatures ranging from 56°C to 65°C. Data from Tt AVG and SD recorded in 500 nm channel for H1N1 synRNA 1x10^4 copies/mL, in 530 nm for influenza B synRNA and in 610 nm for RSV are reported for each temperature tested. Importantly, for each temperature condition tested, primer-dimers% signals in NTC samples were evaluated and reported in the table. According to the results obtained with all three simplex assays, 65°C reaction temperature was selected as best for isothermal amplification due to its amplification speed and absence of primer-dimers signals.

13.5.1.2.Buffer

The buffer regulates the pH of the reaction, which affects the enzyme reverse-transcription and amplification activities. In order to improve RT Q-LAMP reaction, the effect of pH variations on RT Q-LAMP enzyme activity was investigated in a range from 7.8 to 8.8 using influenza A as prototype assay. The buffers used were formulated by DiaSorin and characterized by a proprietary composition. The analysis suggested faster amplification with DiaSorin Buffer with pH 7.8 (Figure 27).

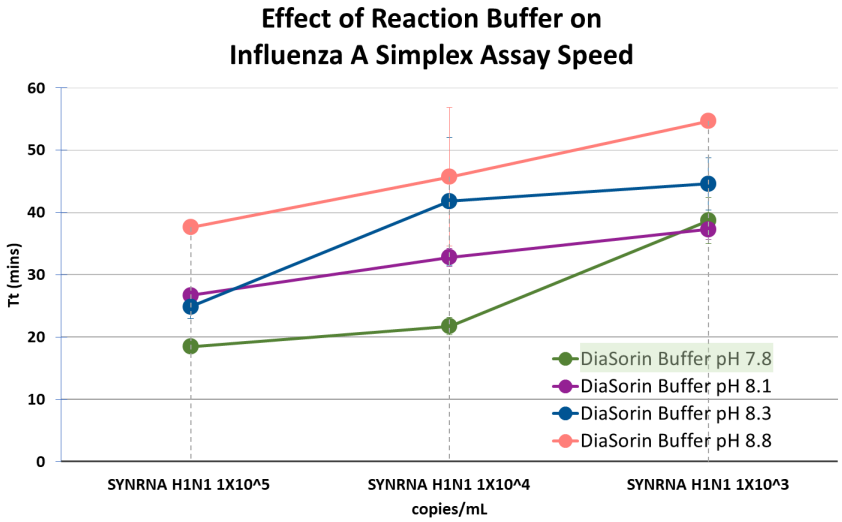
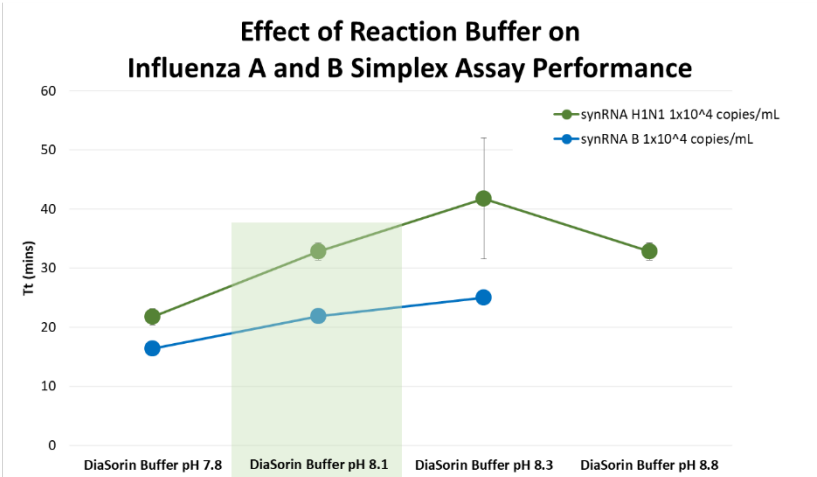


Figure 27. Optimization of RT Q-LAMP influenza A simplex reaction with different buffers. Tenfold serial dilutions of influenza A H1N1 synRNAs ($1x10^5$ – $1x10^4$ – $1x10^3$ copies/mL, assayed in triplicates) were tested with each buffer; AVG and SD Tt recorded in 500 nm channel are reported for each H1N1 SynRNA dose tested. The analysis suggested faster amplification with DiaSorin Buffer pH 7.8.

After preliminary evaluation on influenza A RT Q-LAMP simplex assay, the reaction buffer evaluation was performed also with influenza B simplex assay. Based on preliminary data on influenza A, influenza B assay was tested with pH buffers ranging from 7.8 to 8.3. According to the results obtained, DiaSorin

Buffer pH 7.8 was not chosen for final formulation due primer-dimers amplification observed. Conversely, buffer pH 8.1 displayed best performance in terms of specificity and slightly slower amplifications of approx. 5 minutes. Therefore, it was selected to be included in RT Q-LAMP A, B and RSV quadruplex final assay (Figure 28).



Influenza A	**33%**	**0%**	**0%**	**0%**	**Primer-dimers %**
Influenza B	**33%**	**0%**	**0%**	**0%**	

Figure 28. Influenza A and B RT Q-LAMP simplex assays optimization with different reaction buffers. Buffer evaluation was performed with both influenza A and influenza B simplex assays. Data from Tt AVG and SD recorded in 500 nm channel for H1N1 synRNAs 1x10^4 copies/mL and 530 nm for 1x10^4 copies/mL influenza B synRNA are reported for each buffer tested. In the table below, primer-dimers % is reported for each buffer tested with both assays. DiaSorin Buffer pH 7.8 showed faster performance but also primer-dimers amplification when tested. On contrary, Buffer pH 8.1 displayed best performance in terms of specificity and it was selected to be included in RT Q-LAMP A, B and RSV quadruplex final assay.

13.5.1.3.Magnesium

Since free Mg^{2+} availability affects primers annealing and enzyme activity, the effect of $MgSO_4$ concentrations on the influenza A simplex reaction was determined. Tenfold serial dilutions of influenza A H1N1 synRNAs (in

triplicates) were tested with different $MgSO_4$ concentrations in reaction mix. As expected, decreasing the $MgSO_4$ amount in the reaction resulted in a delay of the amplification Tt up to complete inhibition observed with 1 mM $MgSO_4$ condition (Figure 29).

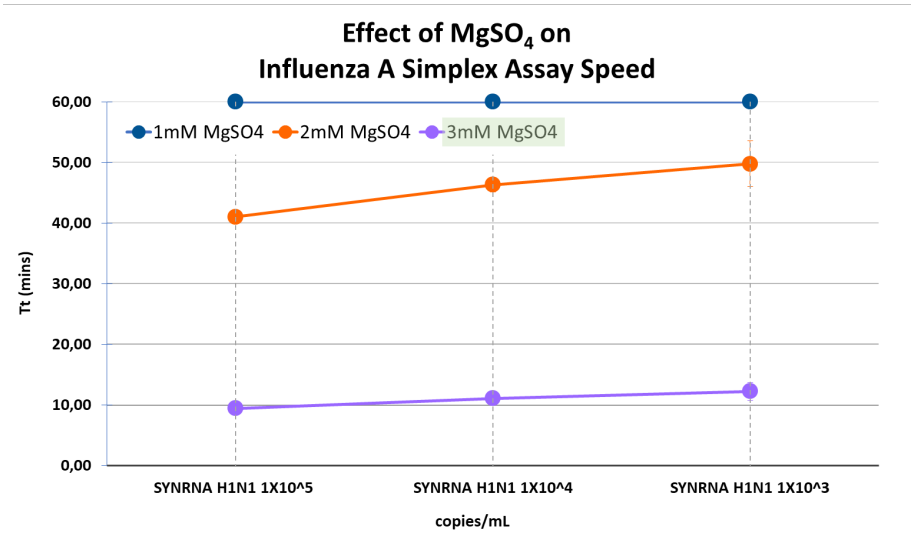
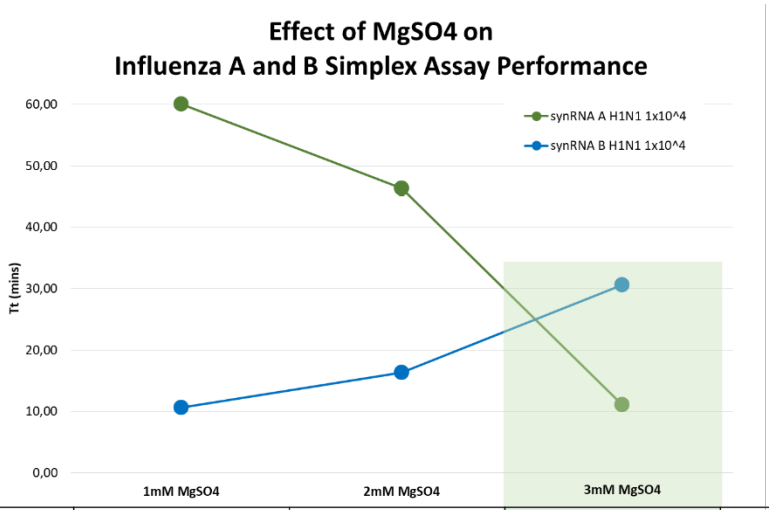


Figure 29. Optimization of RT Q-LAMP influenza A simplex assay with different $MgSO_4$ concentrations. Tenfold serial dilutions of influenza A H1N1 synRNAs ($1x10^5$ – $1x10^4$ – $1x10^3$ copies/mL) were tested with different $MgSO_4$ concentrations in reaction mix. AVG and SD Tt recorded in 500 nm channel are reported for each H1N1 SynRNA dose tested with different $MgSO_4$ concentrations; importantly, amplification at 60 Tt reported in the graph (blue line) means no samples amplification. The analysis showed no amplification with 1 mM $MgSO^4$ and the faster amplification with 3 mM $MgSO_4$.

After preliminary evaluation on influenza A RT Q-LAMP simplex assay, $MgSO_4$ concentration evaluation was performed also with influenza B simplex assay. Different performance was observed with influenza A and B assays: influenza A amplification speeded up increasing $MgSO_4$ concentration, while influenza B amplification slowed down. Consequently, 3mM $MgSO_4$, giving more similar influenza A and B amplification, without any non-specific signal

in NTC, was selected as best condition to be included in RT Q-LAMP A, B and RSV quadruplex final assay (Figure 30).



Influenza A	**66%**	**0%**	**0%**	**Primer-dimers %**
Influenza B	**0%**	**0%**	**0%**	

Figure 30. Influenza A and B RT Q-LAMP simplex assays optimization with different $MgSO_4$ concentrations in the reaction mix: $MgSO_4$ evaluation was performed with both influenza A and B simplex assays. Data from AVG and SD Tt recorded in 500 nm channel for H1N1 synRNAs $1x10^4$ copies/mL and 530 nm for $1x10^4$ copies/mL influenza B synRNA are reported for each $MgSO_4$ concentration tested. In the table below, primer-dimers % is reported for each condition tested with both assays. A concentration of 3 mM $MgSO_4$ was selected as best condition due its specificity and amplification speed, similar between influenza A and B assays. Consequently, it was included in RT Q-LAMP A, B and RSV quadruplex final assay.

13.5.1.4.Deoxynucleotide triphosphate (dNTPs)

A premixed, balanced mixture of dATP, dGTP, dCTP and dTTP was used in the simplex RT Q-LAMP development.

RT Q-LAMP influenza A assay was tested in the presence of several concentrations of dNTPs ranging from 0.6 to 1.6 mM. Similarly to other experimental set up, a preliminary evaluation was performed testing serial

dilutions of synRNAs H1N1 ($1x10^5$ – $1x10^4$ –$1x10^3$ copies/mL in triplicates) at different dNTPs concentration. As result, the dNTPs concentrations between 0.8 and 1.6 mM amplified the target synRNA. In particular, we observed a speeding up of the reaction increasing dNTPs concentration even if not significant differences in terms of speed were observed between 1.2 – 1.4 - 1.6 mM. Using 0.6 mM, no amplification was observed (Figure 31).

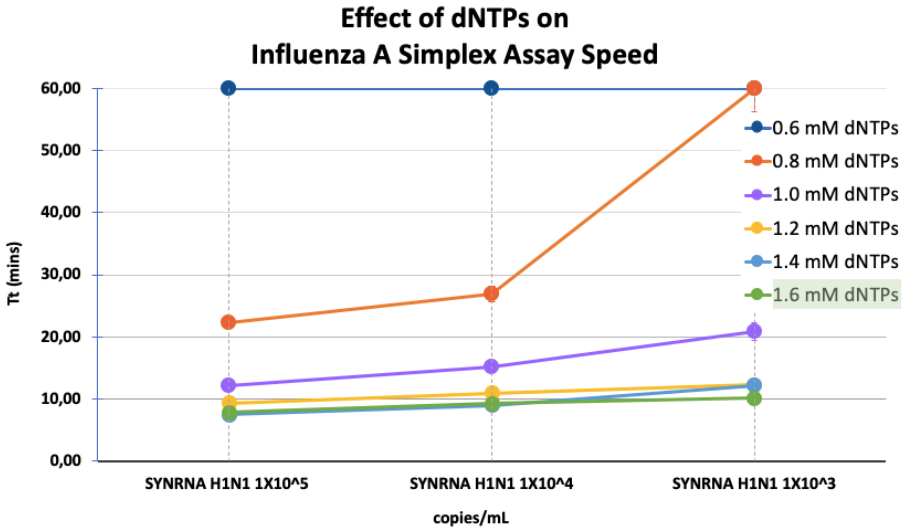
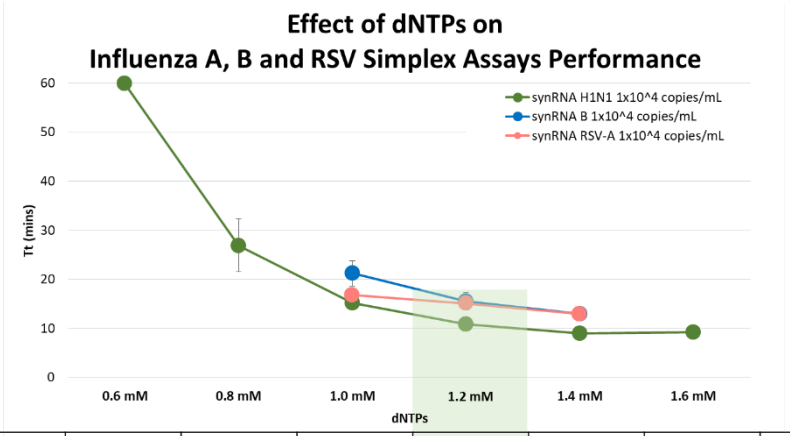


Figure 31. Optimization of RT Q-LAMP influenza A simplex reaction with different dNTPs concentrations ranging from 0.6 mM to 1.6 mM: tenfold serial dilutions of influenza A H1N1 synRNAs ($1x10^5$ – $1x10^4$- $1x10^3$ copies/mL in triplicates) were tested with different dNTPs dose in reaction mix. AVG and SD Tt recorded in 500 nm channel are reported for each H1N1 SynRNA dose tested with different dNTPs amount. dNTPs concentrations between 0.8 mM and 1.6 mM amplified target synRNAs doses. In particular, we observed a speeding up of the reaction increasing dNTPs concentration.

After preliminary evaluation on influenza A RT Q-LAMP simplex assay, dNTPs evaluation was performed also with influenza B and RSV simplex assays. In details, Influenza B and RSV synRNAs $1x10^4$ copies/mL were assayed only with dNTPs ranging from 1.0 mM to 1.4 mM. We observed a slight speed

up trend at the increasing of dNTPs concentration but using more than 1.2 mM of dNTPs, non-specific amplifications were detected in NTC samples. Therefore, 1.2 mM dNTPs concentration was selected as final to be tested with influenza A, B and RSV quadruplex assay (Figure 32).



Influenza A	**0%**	**0%**	**0%**	**0%**	**66%**	**100%**	**Primer-dimers %**
Influenza B	**n.a.**	**n.a.**	**0%**	**0%**	**33%**	**n.a.**	
RSV	**n.a.**	**n.a.**	**0%**	**0%**	**0%**	**n.a.**	

Figure 32. Influenza A, B and RSV RT Q-LAMP simplex assays optimization with dNTPs concentration in the reaction mix. After preliminary evaluation on influenza A RT Q-LAMP simplex assay, dNTPs evaluation was performed also with influenza B and RSV simplex assays. For each dNTPs condition, AVG and SD Tt recorded in 500 nm channel for 1x10^4 copies/mL H1N1 synRNAs, in 530 nm for influenza B and in 610 nm for RSV are reported. Influenza B and RSV assays were tested only with dNTPs ranging from 1.0 mM to 1.4 mM. 1.2 mM dNTPs concentration was selected as best in terms of speed and specificity for all the targets tested and was selected to be tested with influenza A, B and RSV quadruplex assay.

13.5.1.5.Specimen processing step on Liaison MDX

Maintenance of reaction isothermal condition (65°C for 60 minutes) initially could hindered the amplification of the virus (Figure 33). This suggested us the necessity to introduce a preliminary high-temperature step in the thermal protocol. During this step, thanks to DAD design, inside Liaison

MDX thermocycler, the specimen, not yet in contact with the reaction mix, is heated to high temperatures and subjected to a considerable centrifugal force, allowing the release of the viral genome, without the need to preliminarily proceed with RNA extraction.

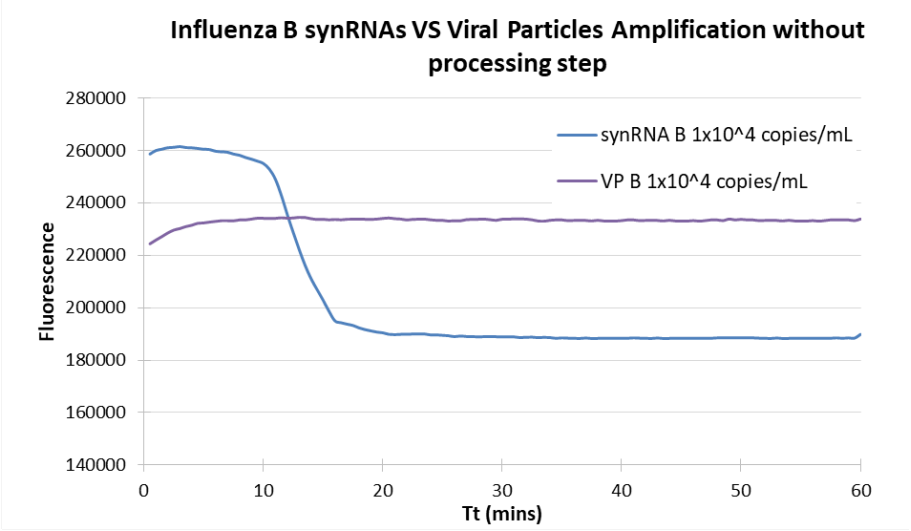


Figure 33. Influenza B synRNAs and viral particles (VPs) amplification in simplex condition without Liaison MDX instrument processing step. Inactivated influenza B viral particles 1x10^4 copies/mL (1 replicate) diluted in PBS were assayed with isothermal condition (65°C for 60 minutes) compared to the same dose of synRNA. No amplification was observed and this suggested us the necessity to introduce a preliminary high-temperature processing step in the thermal protocol.

Seven different thermal pre-treatments were tested on three replicates of 1x10^4 copies/mL of influenza A or B viral particles. Influenza A viruses displayed similar Tt amplification among different protocols while influenza B testing showed great variability between different thermal treatment conditions (Table 6). For this reason, we selected the best protocol for influenza B (which is 98°C for 5 minutes) as the best treatment step for both influenza A and B simplex assays.

Effect of Liaison MDX processing step on Influenza A, B and RSV Simplex Assays						
Temperature (°C)	Influenza A H1N1 VPs 1x10^4 copies/mL			Influenza B VPs 1x10^4 copies/mL		
	Reps detected	AVG Tt	SD Tt	Reps detected	AVG Tt	SD Tt
75°C for 3 mins	n.a.	n.a.	n.a.	3/3	24,7	3,24
75°C for 5 mins	n.a.	n.a.	n.a.	3/3	16,63	2,86
80°C for 3 mins	3/3	19,95	2,1	3/3	27,68	2,93
80°C for 5 mins	2/3	19,2	1,8	3/3	19,13	7,48
98°C for 3 mins	2/3	18,52	1,92	3/3	21,3	1,2
98°C for 5 mins	3/3	17,95	0,59	3/3	16,67	0,09
98°C for 10 mins	1/3	18,25	--	1/3	16,05	--

Table 6. Liaison MDX processing step optimization on influenza A and B simplex assays: seven different thermal pre-treatments were tested on three replicates of 1x10^4 copies/mL of influenza A or B viral particles. In the table, number of replicates detected, Tt AVG and SD for each condition tested are reported. In details, influenza B testing showed great variability between different thermal treatment conditions. Otherwise, influenza A viruses displayed similar Tt amplification among different protocols. Accordingly, we selected 98°C for 5 minutes pre-treatment step as best on influenza A and B simplex assays.

After preliminary viral particles processing evaluation with influenza A and B simplex assays, we selected conditions at 98°C (98°C for 3 minutes, 98°C for 5 minutes and 98° for 10 minutes) to be tested with 1x10^4 copies/mL of viral particles in triplicates (influenza A H1N1, influenza A H3N2, influenza B, RSV-A or RSV-B) on Liaison MDX instrument. As shown in Figure 34, 98°C 5 minutes treatment confirmed to be the most promising in terms of viral particles amplification speed and precision. According to the analysis, we concluded that both 3 and 10 minutes treatments might not be optimal due to a significant delay especially in RSV-B viral particles amplification. Therefore, the pre-treatment at 98°C for 5 minutes was confirmed as the best condition.

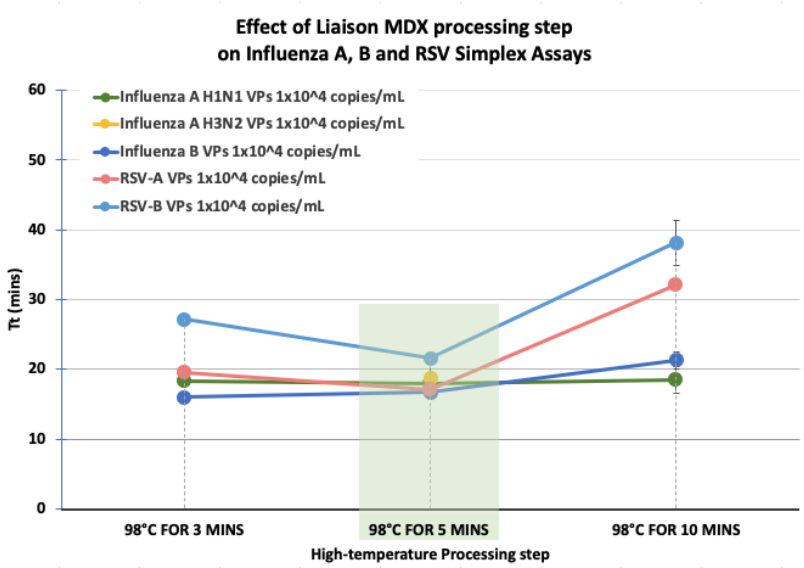


Figure 34. Effect of Liaison MDX instrument processing step on influenza A, B and RSV simplex assays. After preliminary viral particles processing evaluation with influenza A and B simplex assays, we selected conditions at 98°C (98°C for 3 minutes, 98°C for 5 minutes and 98° for 10 minutes) to be tested with triplicates of $1x10^4$ copies/mL viral particles (influenza A H1N1, influenza A H3N2, influenza B, RSV-A and RSV-B) on Liaison MDX instrument. As graphically represented, 98°C 5 minutes treatment confirmed to be the most promising in terms of viral particles amplification speed and precision.

In summary, the final thermal protocol selected for amplification of influenza A, B and RSV viruses with RT Q-LAMP simplex and quadruplex assays was 98°C for 5 minutes followed by 65°C isothermal amplification, as reported in Table 7.

Influenza AB & RSV Assay Thermal Protocol	
Sample	**Rx Mix**
98°C for 5 mins	--
Mixing (1 min)	
65°C for 50 mins (capture every 30 sec)	

Table 7. Schematic representation of influenza A, B and RSV final Liaison MDX thermal protocol used for amplification of influenza A, B and RSV viruses with RT Q-LAMP simplex and quadruplex assays.

13.5.2.Influenza A, B and RSV quadruplex assay reaction optimization

13.5.2.1.Primers concentration

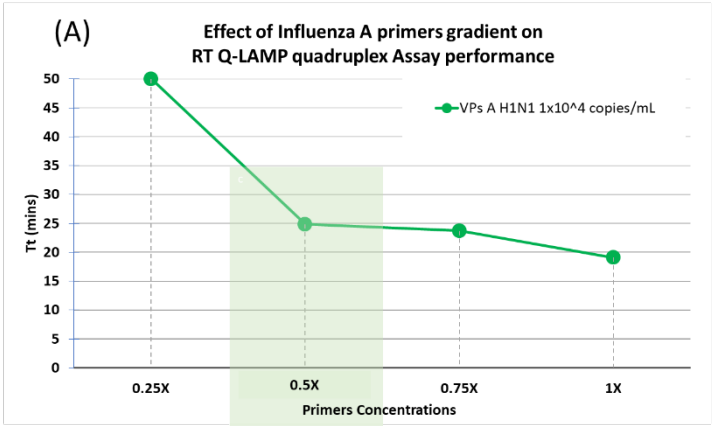
Influenza A, B and RSV RT Q-LAMP quadruplex reaction mix obtained is composed by mixing all the four primers sets involved in the simplex reactions. Starting from 1X standard concentrations for all the primers (reported in Materials and Methods section), we tested $1x10^4$ copies/mL of inactivated virus stocks with new conditions, changing both influenza A, B and RSV primers concentrations. In particular, a detailed fine-tuning process was followed, changing each single primer involved in the reaction (data not shown).

Best primers condition was defined according to absence of primer-dimers in NTC and faster detection of viruses amplification.

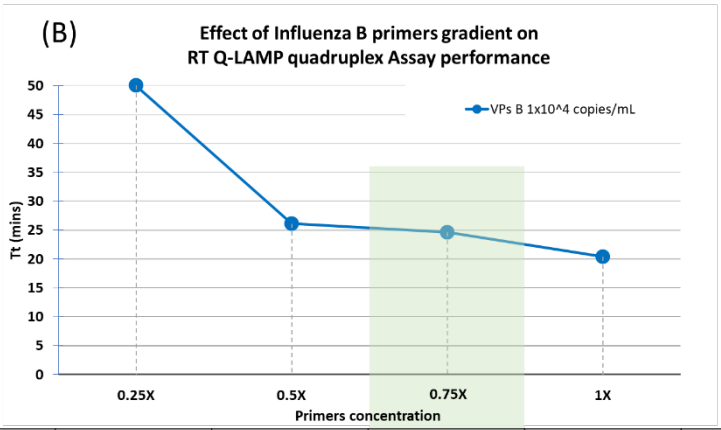
In detail, for influenza A and B amplification, we observed similar performance between 0.5X, 0.75X and 1X, characterized by a speed up in the reaction when increasing primers amount. We did not observe any amplification with 0.25X concentration (Figure 35A, B). Importantly, a significant increase in primer-dimers detection with 0.75X and 1X influenza A primers amount was observed. Therefore, we selected 0.5X and 0.75X amount as final condition for influenza A and B primers respectively (Table 8).

RSV amplification showed faster performance with 1X condition compared to 0.75X. With less than 0.75X primers, amplification was completely lost.

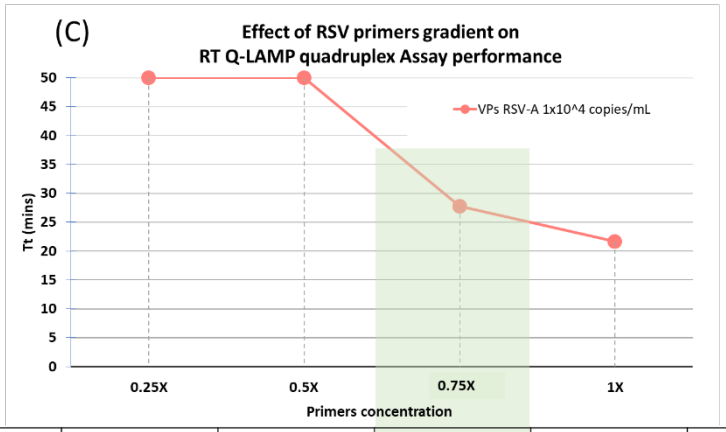
Similarly to influenza B primers behavior, primer-dimers signals were observed only with 1X condition. Consequently, we selected RSV 0.75X primers amount for quadruplex testing (Figure 35C).



Influenza A	0%	0%	16%	33%	Primer-dimers %



Influenza B	0%	0%	0%	16%	Primer-dimers %



RSV	0%	0%	0%	33%	Primer-dimers %

Figure 35. Reaction primers concentration optimization with influenza A, B and RSV RT Q-LAMP quadruplex assay: Tt AVG and SD recorded in 500 nm channel for 1x10^4 copies/mL H1N1 virus (tested in triplicates), 530 nm for influenza B and 610 nm for RSV are reported.

In summary, primers concentrations selected for final RT Q-LAMP assay were 0.5X, 0.75X and 0.75X for influenza A, B and RSV respectively (Table 8).

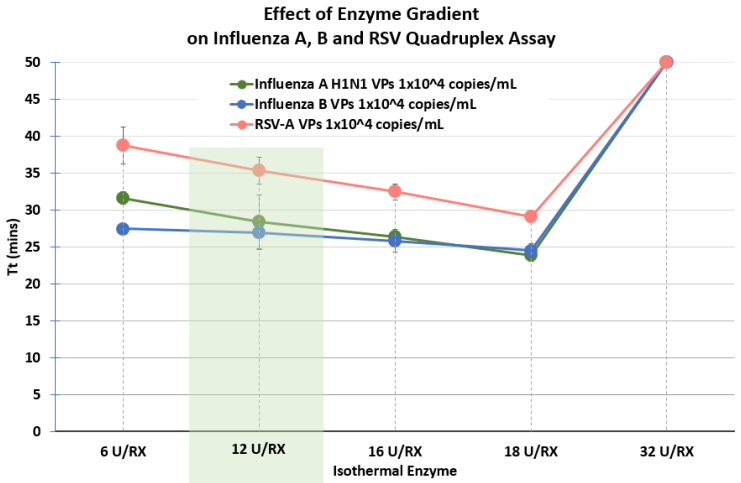
Primers concentration fine-tuning	
Simplex Assay	**Final condition selected**
Influenza A	0.5X
Influenza B	0.75X
RSV	0.75X

Table 8. Final primers concentrations condition selected with influenza A, B and RSV quadruplex reaction: the primers concentration evaluation process led to the selection of all final primers conditions to be used in quadruplex reaction. We selected 0.75X primers for both influenza B and RSV and 0.5X for influenza A target.

13.5.2.2.Enzyme concentration

During the quadruplex optimization, different concentrations of enzymes were tested. In the standard LAMP condition, the concentration of the enzyme is 8 U/rx. (Figure 36).

Different concentrations around this value were tested: increasing the enzyme units per reaction (U/rx) in the reaction mix, we can detect earlier the viral particles 1x10^4 copies/mL, even if too high concentrations (32 U/rx) inhibited the reaction. Importantly, although the conditions with 16 U/rx and 18 U/rx showed faster viral particles amplification, they were excluded because characterized by a greater detection of primer-dimers (Figure 36). Therefore, 12 U/rx condition was selected as best for influenza A, B and RSV final quadruplex assay performance.



Influenza A	0%	0%	0%	16%	0%	Primer-dimers %
Influenza B	0%	0%	16%	16%	33%	
RSV	0%	0%	0%	0%	16%	

Figure 36. Effect of isothermal enzyme gradient on influenza A, B and RSV quadruplex assay: data from amplification of viral particles 1x10^4 copies/mL are reported for each enzyme condition tested; Tt AVG and SD recorded in 500 nm

channel for H1N1 synRNAs, 530 nm for influenza B and 610 nm for RSV are reported. In the table below, primer-dimers % is reported for each condition tested: different enzyme concentrations were tested, finding a slight increase of reaction speed at higher concentrations for all the targets. Unfortunately, in parallel to the speed up, an increase of primer-dimers and non-specific signals was detected.

13.6.IC amount definition and evaluation

In the final assay set up, inactivated MS2 bacteriophage is included in the reaction mix. Its addition is important to verify the effectiveness of the reverse-transcription and amplification of the viral nucleic acid. To be noted, according to our detection algorithm, described in Materials and Methods section, IC amplification is not a critical feature in samples resulting positive for influenza A, B or RSV targets but it has to occur at least in negative samples, in order to validate the results.

To find the most appropriate amount of MS2 phage to include in the reaction mix, tenfold serial dilutions ($1x10^3$, $1x10^2$, 10 copies/reaction) were added directly into the quadruplex format reaction mix. Seven replicates of NTC samples (PBS) were tested for each MS2 Bacteriophage dose in order to choose the amount to be included in the reaction mix giving 100% amplification around 30 Tt: amplification faster than 30 Tt could potentially run out of available reagents before the viral target amplification.

According to the results obtained, we observed that 10 copies/reaction dose was 71% (5/7 reps) detected in NTC and therefore excluded. Both 10^3 and 10^2 copies/reaction doses were 100% (7/7) detected in NTC. Higher amount of IC (10^3 copies/reaction) could potentially run out available

reagents during PCR before the viral target amplification and it was not chosen as final dose.

Therefore, we selected 10^2 copies/reaction as final dose to be included in our mix, being an effective indicator of proper sample reverse-transcription and amplification (Table 9).

MS2 phage amount in Influenza A, B and RSV Quadruplex Assay									
Sample	MS2 Phage $1x10^3$ copies/rx			MS2 Phage $1x10^2$ copies/rx			MS2 Phage 10 copies/rx		
	Reps detected	AVG Tt	SD Tt	Reps detected	AVG Tt	SD Tt	Reps detected	AVG Tt	SD Tt
NTC (PBS)	7/7	19,25	0,34	7/7	25,33	0,06	5/7	34,25	3,12

Table 9. IC (MS2 Bacteriophage) serial dilutions in influenza A, B and RSV quadruplex assay. Tenfold serial dilutions of inactivated MS2 Bacteriophage in the influenza A, B and RSV reaction mix were evaluated testing NTC samples with the quadruplex format. Three different doses ($1x10^3$ copies/reaction; $1x10^2$ copies/reaction; 10 copies/reaction) were assayed. According to these testing, we selected the dose giving the slowest amplification to which we had 100% detection in NTC, so as not to compete with the target amplification, being an effective indicator of proper sample reverse-transcription and amplification.

14. INFLUENZA A, B AND RSV QUADRUPLEX ASSAY ANALYTICAL PERFORMANCE

14.1. Analytical sensitivity

Analytical sensitivity is determined by the Limit of Detection (LoD) value, which represents the lowest amount of target that can be detected in at least 95% of replicates. The performance of the influenza A, B and RSV quadruplex assay was evaluated testing serial dilutions of influenza A H1N1, H3N2, influenza B and RSV-A2 viral particles in PBS. As reported in Table 10, the final quadruplex assay resulted in detection of at least $1x10^3$ copies/mL of

inactivated virus diluted in PBS in 95% of cases for both influenza A and B and RSV targets, showing a good sensitivity.

Influenza A, B & RSV Analytical Sensitivity				
Sample (copies/mL)	**Flu A H1N1 Detection (reps)**	**Flu A H3N2 Detection (reps)**	**Flu B Detection (reps)**	**RSV Detection (reps)**
1x10^5	100% (40/40)	100% (40/40)	100% (40/40)	100% (40/40)
1x10^4	100% (40/40)	100% (40/40)	100% (40/40)	100% (40/40)
1x10^3	97.5% (39/40)	95% (38/40)	97.5% (39/40)	95% (38/40)
1x10^2	70% (28/40)	65% (26/40)	75% (30/40)	72.5% (29/40)

Table 10. Analytical sensitivity of influenza A, B and RSV quadruplex assay. Analytical sensitivity is determined by the Limit of Detection (LoD) value, which represents the lowest amount of target that can be detected in at least 95% of replicates. According to the analysis performed, we concluded that the LoD of influenza A, B and RSV RT Q-LAMP assay is $1x10^3$ copies/mL.

14.2.Analytical specificity

Analytical specificity is defined as absence of non-specific signal in NTC samples. For influenza A, B and RSV RT Q-LAMP assay, it was assessed on 100 replicates of NTC, represented by both DEPC-treated water and PBS. In details, 50 replicates of DEPC-treated water and 50 replicates of PBS were tested giving 100% specificity without primer-dimers detected in any of the instrument's target amplification channels (500 nm, 530nm, 610nm). All results were validated through the correct amplification of the IC in 682 nm channel, which excludes the presence of inhibitors and ensures the use of correct reaction conditions (Table 11). Moreover, to validate the results, in each run $1x10^4$ copies/mL of influenza A, B and RSV inactivated virus mixture diluted in PBS were included (data not shown).

Influenza A, B and RSV Analytical Specificity						
Channel	NTC (PBS)			NTC (Water)		
	Reps detected	AVG Tt	SD Tt	Reps detected	AVG Tt	SD Tt
500 nm channel	0/50	--	--	0/50	--	--
530 nm channel	0/50	--	--	0/50	--	--
610 nm channel	0/50	--	--	0/50	--	--
682 nm channel	50/50	26.2	3.8	50/50	24.3	2.1

Table 11. Analytical specificity of RT Q-LAMP influenza A, B and RSV quadruplex assay. Fifty replicates of DEPC-treated water and 50 replicates of PBS were tested giving 100% specificity without primer-dimers detected in any of the instrument's target amplification channels (500 nm, 530nm, 610nm). All results were validated through the correct amplification of the IC in 682 nm channel.

14.3.Analytical reactivity

Analytical reactivity is the ability of our RT Q-LAMP assay to detect and amplify different influenza A, B and RSV viral strains circulating from geographically diverse locations. It was evaluated using quantified viral material spiked into PBS at the concentrations reported in Table 12 and assayed in triplicate.

As reported, 100% influenza A strains tested were detected. As one H1N2 and one H9N2 strain subtypes were also available in our laboratories they were tested: even in this case influenza A, B and RSV RT Q-LAMP assay showed its ability to detect them.

Organisms (Influenza A viruses)	Tested Concentration (1)	% Detection
A/California/4/2009 (H1N1)	100 TCID50/mL	100%
A/California/12/2012 (H1N1)	100 TCID50/mL	100%
A/Massachusetts/15/2013 (H1N1)	1000 CEID50/mL	100%
A/Mexico/4108/2009 (H1N1)	100 CEID50/mL	100%
A/New York/18/2009 (H1N1)	100 CEID50/mL	100%
A/Brisbane/59/07 (H1N1)	100 TCID50/mL	100%
A/Hawaii/15/2001 (H1N1)	100 CEID50/mL	100%
A/New Caledonia/20/99 (H1N1)	100 TCID50/mL	100%
A/Solomon Island/03/06 (H1N1)	100 TCID50/mL	100%
A/Taiwan/42/06 (H1N1)	100 TCID50/mL	100%
A/WS/33 (H1N1)	100 TCID50/mL	100%
A/Brisbane/10/07 (H3N2)	100 TCID50/mL	100%
A/California/02/2014 (H3N2)	100 CEID 50/mL	100%
A/New York/55/2004 (H3N2)	100 CEID50/mL	100%
A/Ohio/02/2012 (H3N2)	200 CEID50/mL	100%
A/Port Chalmers/1/73 (H3N2)	100 TCID50/mL	100%
A/Rhode Island/01/2010 (H3N2)	400 CEID50/mL	100%
A/Santiago/7981/2006 (H3N2)	100 CEID50/mL	100%
A/Switzerland/9715293/2013 (H3N2)	200 CEID50/mL	100%
A/Texas/50/2012 (H3N2)	100 CEID 50/mL	100%
A/Wisconsin/67/05 (H3N2)	100 TCID50/mL	100%
A/Indiana/08/2011 (H3N2)	100 CEID 50/mL	100%
A/Minnesota/11/2010 (H3N2)	100 CEID50/mL	100%
A/Hong Kong/33982/2009(H9N2)-PR8-IDCDC_RG26	100 CEID50/mL	100%
A/Singapore/INFIMH-16-0019/2016 (H3N2)	100 CEID50/mL	100%
A/Michigan/45/2015 (H1N1)	100 CEID50/mL	100%
A/Minnesota/19/2011 (H1N2)	1000 CEID50/mL	100%
A/Hong Kong/4801/2014 (H3N2)	200 CEID50/mL	100%
A/Kansas/14/2017 (H3N2)	100 EID50/mL	100%
A/Brisbane/02/2018 (H1N1)	100 EID50/mL	100%
A/Christchurch/16/2010 (H1N1)	200 EID50/mL	100%
A/Hong Kong/2671/2019 (H3N2)	100 EID50/mL	100%
A/Guandong-Maonan/1536/2019 (H1N1)	100 EID50/mL	100%
A/Perth/16/2009 (H3N2)	100 EID50/mL	100%
A/NY/02/09 8H1N1)	0.04 TCID50/mL	100%

(1) TCID50/mL = Tissue Culture Infectious Dose
CEID50/mL = Chicken Embryo Infectious Dose
EID50/mL = Egg Infectious Dose

Table 12. *In vitro* analytical reactivity of RT Q-LAMP influenza A, B and RSV quadruplex assay on influenza A viruses. Analytical reactivity was evaluated using quantified viral material spiked into PBS at the concentrations reported and assayed in triplicate. Thirty-five influenza A strains were evaluated. Common circulating strains from geographically diverse locations were chosen. All strains tested were detected.

Analytical reactivity was subsequently evaluated testing influenza B strains from geographically diverse locations. Similarly to influenza A, influenza B quantified viral material was spiked into PBS at the concentrations reported in Table 13 and assayed in triplicate.

Thanks to this evaluation, we concluded that influenza A, B and RSV RT Q-LAMP assay is able to detect all 25 influenza B strains tested.

Organisms (Influenza B viruses)	Tested Concentration (1)	% Detection
B/Brisbane/33/2008	20 CEID50/mL	100%
B/Brisbane/60/2008	20 CEID50/mL3	100%
B/Florida/02/2006	100 TCID50/mL	100%
B/Lee/40	100 TCID50/mL	100%
B/Nevada/03/2011	100 CEID50/mL	100%
B/Texas/02/2013	100 TCID50/mL	100%
B/Victoria/304/2006	50 CEID50/mL	100%
B/Christchurch/33/2004	100 CEID50/mL	100%
B/Florida/07/04	100 TCID50/mL	100%
B/Florida/04/2006	100 TCID50/mL	100%
B/Guangdong-Liwan/1133/2014	400 CEID50/mL	100%
B/Maryland/1/59	100 TCID50/mL	100%
B/Great Lakes/1739/54	100 TCID50/mL	100%
B/Panama/45/90	100 TCID50/mL	100%
B/Phuket/3073/2013	100 CEID50/mL	100%
B/Utah/9/2014	100 CEID50/mL	100%
B/Wisconsin/01/2010	100 CEID50/mL	100%
B/Allen/45	100 TCID50/mL	100%
B/Hong Kong/5/72	100 TCID50/mL	100%
B/Taiwan/2/62	100 TCID50/mL	100%
B/Colorado/06/2017	20 TCID50/mL	100%
B/Michigan/09/2011	100 EID50/mL	100%
B/Texas/81/2016	20 EID50/mL	100%
B/Washington/02/2019	100 EID50/mL	100%
B/New Hampshire/01/2016	100 EID50/mL	100%

(1) TCID50/mL = Tissue Culture Infectious Dose
CEID50/mL = Chicken Embryo Infectious Dose
EID50/mL = Egg Infectious Dose

Table 13. *In vitro* analytical reactivity of RT Q-LAMP influenza A, B and RSV quadruplex assay on influenza B viruses: analytical reactivity was evaluated using quantified viral material spiked into PBS at the concentrations reported and assayed in triplicate. Twenty-five influenza B strains were evaluated. Common

circulating strains from geographically diverse locations were chosen. All strains tested were detected.

Finally, we tested different RSV available viruses diluted in PBS and tested in triplicates. Also in this case, influenza A, B and RSV RT Q-LAMP assay detected all 11 RSV tested (Table 14).

Organisms (RSV)	Tested Concentration (1)	% Detection
ATCC-2012-10	100 PFU/mL	100%
A 1997/12-35	100 TCID50/mL	100%
A 1998/12-21	100 TCID50/mL	100%
A 1998/3-2	100 TCID50/mL	100%
A 2000/3-4	100 TCID50/mL	100%
A 2001/2-20	100 TCID50/mL	100%
A 2001/3-12	100 TCID50/mL	100%
A Long	100 TCID50/mL	100%
B 9320	100 TCID50/mL	100%
B/Wash/18537/62	100 TCID50/mL	100%
B/WV/14617/85	100 TCID50/mL	100%

(1) TCID50/mL = Tissue Culture Infectious Dose
PFU/mL = Plaque forming units

Table 14. *In vitro* analytical reactivity of RT Q-LAMP influenza A, B and RSV quadruplex assay on RSV. Analytical reactivity was evaluated using quantified viral material spiked into PBS at the concentrations reported and assayed in triplicate. Eleven RSV (A and B subtypes) were evaluated. Common circulating strains from geographically diverse locations were chosen. All strains tested were detected.

14.4.Cross-reactivity

Cross-reactivity is defined as the ability of our RT Q-LAMP to detect organisms that are closely related, or cause similar clinical symptoms, or present as normal flora in NPS specimens. Therefore, the goal was to verify that influenza A, B and RSV RT Q-LAMP assay exclusively identified influenza A virus and/or influenza B virus and/or RSV. In detail, cross-reactivity was tested with 26 different bacteria and viruses and assayed in triplicate and no

cross-reactivity was observed with the 26 organisms. The organisms, the concentration at which these organisms were tested and the results obtained are reported in Table 15.

Influenza AB and RSV Cross-reactivity					
Organism	Tested Concentration (1)	% Detection			
		500 nm channel	560 nm channel	610 channel	682 channel
Adenovirus Type 1	1x10^6 TCID50/mL	0%	0%	0%	100%
Adenovirus Type 7A	1x10^5 TCID50/mL	0%	0%	0%	100%
Bordetella pertussis A639	1x10^7 CFU/mL	0%	0%	0%	100%
Chlamydia pneumoniae	1x10^6 IFU/ml	0%	0%	0%	100%
CMV 169	1x10^5 TCID50/mL	0%	0%	0%	100%
Coronavirus 229E	1x10^5 TCID50/mL	0%	0%	0%	100%
Coronavirus OC43	1x10^5 TCID50/mL	0%	0%	0%	100%
Coronavirus NL63	1x10^5 TCID50/mL	0%	0%	0%	100%
Enterovirus Type 71	1x10^5 TCID50/mL	0%	0%	0%	100%
Epstein Barr Virus	1x10^6 copies/mL	0%	0%	0%	100%
Escherichia coli	1x10^7 CFU/mL	0%	0%	0%	100%
Haemophilus influenzae	1x10^7 CFU/mL	0%	0%	0%	100%
Metapneumovirus 9	1x10^6 TCID50/mL	0%	0%	0%	100%
Mycobacterium tuberculosis (genomic DNA)	1x10^7 copies/mL	0%	0%	0%	100%
Mycoplasma pneumoniae	1x10^6 CCU/mL	0%	0%	0%	100%
Neisseria meningitidis	1x10^6 CFU/mL	0%	0%	0%	100%
Parainfluenza 1	1x10^5 TCID50/mL	0%	0%	0%	100%*
Parainfluenza 2	1x10^6 TCID50/mL	0%	0%	0%	100%
Parainfluenza 3	1x10^5 TCID50/mL	0%	0%	0%	100%
Pseudomonas aeruginosa	1x10^7 CFU/mL	0%	0%	0%	100%
Rhinovirus 1A	1x10^5 TCID50/mL	0%	0%	0%	100%
SARS-CoV2	1x10^6 TCID50/mL	0%	0%	0%	100%
Staphylococcus aureus, COL	1x10^7 CFU/mL	0%	0%	0%	100%
Streptococcus pneumoniae	1x10^7 CFU/mL	0%	0%	0%	100%
Streptococcus pyogenes, M1	1x10^7 CFU/mL	0%	0%	0%	100%
Streptococcus salivarius	1x10^7 CFU/mL	0%	0%	0%	100%

*One invalid replicate (IC failure) was repeated and valid.
(1) TCID50/mL = Tissue Culture Infectious Dose
CFU/mL = Colony Forming Units
IFU/mL = Infectious Units

Table 15. *In vitro* cross-reactivity of RT Q-LAMP influenza A, B and RSV quadruplex assay. Twenty-six different bacteria and viruses were assayed in triplicate. The organisms and the concentration at which these organisms were tested are presented. No cross-reactivity was observed with all the organisms tested.

15. INFLUENZA A, B AND RSV FINAL ASSAY VALIDATION ON CLINICAL SAMPLES

The clinical agreement study was conducted using retrospective pre-selected positive and negative leftover nasal and NPS specimens from human patients with signs and symptoms of respiratory tract infections.

The specimens were collected from Italian and U.S. hospitals from subjects who gave their informed consent. The results assayed with influenza A, B and RSV RT Q-LAMP were compared to those assayed with standard diagnostic RT-PCR methods routinely used in the hospital laboratory. We tested a total of 90 positive clinical specimens: 10 influenza A H1N1 positive, 20 influenza A H3N2 positive, 30 influenza B positive and 30 RSV positive nasal and NPS. In addition, 30 influenza A, B or RSV negative NPS or BAL clinical samples, were tested. The clinical specificity was 100% for all the targets assayed and the clinical sensitivity ranged from 87% (for influenza A and RSV detection) to 90% (for influenza B). Moreover, The Positive Predictive Value (PPV) was 100% while the Negative Predictive Value (NPV) ranged from 96% to 97% (Table 16).

Influenza A		Hospital Diagnosis		
		Positive	Negative	Total
RT Q-LAMP	Detected	26	0	26
	Not Detected	4	90	94
	Total	30	90	120
	PPA	100%	NPA	96%
	Sensitivity	87%	Specificity	100%

Influenza B		Hospital Diagnosis		
		Positive	Negative	Total
RT Q-LAMP	Detected	27	0	27
	Not Detected	3	90	93
	Total	30	90	120
	PPA	100%	NPA	97%
	Sensitivity	90%	Specificity	100%

RSV		Hospital Diagnosis		
		Positive	Negative	Total
RT Q-LAMP	Detected	26	0	26
	Not Detected	4	90	94
	Total	30	90	120
	PPA	100%	NPA	96%
	Sensitivity	87%	Specificity	100%

Table 16. Influenza A, B and RSV RT Q-LAMP validation on clinical samples. We tested a total of 90 retrospective pre-selected positive clinical specimens: 10 influenza A H1N1 positive, 20 influenza A H3N2 positive, 30 influenza B positive and 30 RSV positive nasal and NPS. Thirty influenza A, B or RSV negative NPS or BAL clinical samples, were also tested. Here the comparison between hospital diagnosis and RT Q-LAMP detection is reported: column data represent the diagnosis numbers provided by the hospital; row data represent the detection of RT Q-LAMP assay. For example, for influenza A, 30 specimens were positively diagnosed by the hospital. Among them 26 out of 30 were positively identified by RT Q-LAMP assay, 4 out of 30 were not detected by our assay thus representing false negative results.

DISCUSSION

In the current era of emerging novel respiratory viruses, there is a growing need for rapid, sensitive and specific identification of viral pathogens to allow effective prompt antimicrobial therapy, decrease extra diagnostic testing and implement pathogen-specific infection control measures. It is challenging to clinically distinguish bacterial from viral infections and different viral pathogens (Rebnord et al., 2016). This diagnostic uncertainty leads to overprescription of antibiotics and extra diagnostic testing (along with costs) to rule out bacterial infections (Doan et al., 2014; Shah et al., 2016). Rapid detection of these viral pathogens could overcome these disadvantages and laboratory testing may be useful in hospitalized patients with suspected respiratory infection and in patients for whom a confirmed diagnosis will change treatment decisions. Moreover, during a respiratory illness outbreak in a closed setting (e.g., hospitals, long-term care facility, cruise ship, boarding school, summer camp) testing for influenza virus infection can be very helpful in determining if influenza is the cause of the outbreak.

Therefore, prompt viral diagnosis may lead to rapid implementation of infection control measures, early administration of antiviral medication, if available and shorter hospital stays, resulting in reduced healthcare costs (Bruning et al., 2017; Klepser et al., 2015).

In this context, clinical signs and symptoms of influenza and RSV respiratory tract infections are similar and frequently overlap with those of bacterial infections.

Influenza, commonly known as “flu”, is an infectious respiratory disease caused by influenza viruses. These viruses belong to the *Orthomyxoviridae*, a family of negative-sense RNA viruses.

There are four types of influenza viruses: A, B, C and D but only influenza A and B, can cause annual epidemics of varying severity, including mild common cold symptoms to severe lung injury with fatal outcome. While influenza B viruses are almost exclusively found in humans, influenza A viruses circulate in the human population as an annually recurring epidemic disease and emerge from a huge zoonotic reservoir (Centers for Disease Control and Prevention (CDC), 2020c).

Influenza A is classified further by describing two viral proteins expressed on its surface, hemagglutinin and neuraminidase. Seasonal influenza viruses usually contain one of three major subtypes of hemagglutinin (H1, H2, or H3) and one of two subtypes of neuraminidase (N1 or N2). Influenza B is not classified into subtypes (Petrova & Russell, 2018).

Importantly, influenza A viruses are the only influenza viruses known to cause pandemics. Characterized by their ability to rapidly acquire adaptive mutations in a process called *antigenic drift*, influenza A viruses gradually evade the human immune response (Schmolke & García-Sastre, 2010). Additionally, the special arrangement of viral genetic information on multiple RNA segments allows for mixing genetic information of different influenza A virus strains giving rise to novel, gene-reassorted virus strains, a process called *antigenic shift* that periodically results in emergence of virus strains with largely altered characteristics and the ability to infect immunologically naive humans with increased pathogenicity during pandemic outbreaks.

Influenza B viruses generally change more slowly in terms of their genetic and antigenic properties than influenza A viruses and therefore typically have a slower circulation (Centers for Disease Control and Prevention (CDC), 2019). However, the proportion of influenza B viruses that circulate can vary by geographic location.

Despite advances in diagnostic and treatment strategies, mortality from influenza continues to increase: WHO estimates that seasonal influenza may result in 290,000-650,000 deaths each year due to respiratory diseases alone (World Health Organisation (WHO), 2020).

Respiratory syncytial virus, or RSV, is a common respiratory virus that usually causes mild, cold-like symptoms. Most people recover in a week or two, but RSV can be serious, especially for infants and older adults. In fact, RSV is the most common cause of bronchiolitis and pneumonia in children younger than 1 year of age. It is also a significant cause of respiratory illness in older adults.

In the last years, rapid influenza and RSV diagnostic molecular tests were developed. Compared with other diagnostic modalities—culture, or immunofluorescence testing—molecular tests are often faster, less expensive, easier to use and accessible to staff without laboratory training. They have the potential to be carried out at or near the point of care (Bruning et al., 2017). The majority of these tests require extracting viral genomes to be amplified by multiplex PCR or RT-PCR and this process is time consuming, needing skilled staff and equipped laboratories.

Loop-mediated isothermal AMPlification (LAMP) is a powerful nucleic acids amplification technique, which has emerged as an easy-to-perform and rapid

tool for molecular diagnostics applications in clinical routine (Notomi et al., 2000). LAMP amplifies DNA or RNA target with high specificity, efficiency and rapidity under isothermal conditions. It is fast, as the reaction is usually completed in 60 minutes or less; it is relatively inexpensive thanks to the use of a strand displacement polymerase, avoiding the use of expensive thermocycler instruments. All these peculiarities make LAMP intrinsically different from PCR and allow achieving superior performances in those applications in which the unique characteristics of this method are magnified, as the differential diagnosis of viruses.

In DiaSorin the technology was improved in two main characteristics, giving rise to RT Q-LAMP: the coupling of reverse-transcription and amplification activity in one enzyme, which allowed to start the reaction directly from RNA and in a single tube and the use of fluorescent probes (Q-probes), which allowed the real time detection and discrimination of multiple targets.

Considering all these features, this thesis describes the development of a RT Q-LAMP-based assay targeting conserved regions of influenza A, B and RSV viruses to differentially diagnose these infections. Both reverse-transcription and amplification are performed in a single-step from clinical specimens (represented by both nasal and nasopharyngeal swabs - NPS) that have not undergone nucleic acid extraction. RT Q-LAMP is performed thanks to Liaison MDX thermocycler, used with its consumable disc named “Direct Amplification Disc” (DAD). This disc can run from one to eight samples at a time and allows the amplification of non-extracted samples, thanks to the combination of centrifugal force and heat treatment.

The reaction was optimized using reagents with improved sensitivity and specificity, allowing clinicians to obtain results for a therapeutic intervention in less than 60 minutes.

In detail, influenza A, B and RSV RT Q-LAMP assay consists in a multiplex primer system: the first set (labelled with a FAM fluorophore) targeting influenza A viruses, the second set (labelled with a JOE fluorophore), targeting influenza B viruses, the third set (labelled with a CFR610 fluorophore), targeting RSV-A and RSV-B and the fourth set (labelled with Q670 fluorophore) detecting and amplifying an Internal Control (IC) target. The use of the IC in RT Q-LAMP is very important to control the sample reverse-transcription and amplification and to evaluate the presence of reaction inhibitors allowing to validate negative results. It is made from inactivated MS2 bacteriophage particles that are included into reaction mix.

After a long effort of reaction optimization, the most promising formulation was selected.

The final formulation was tested in terms of analytical performances: no false positives were detected on over 100 replicates of NTCs represented by both 50 replicates of DEPC-treated water and 50 replicates of PBS. The analytical sensitivity is determined by the Limit of Detection (LoD) value, which represents the lowest amount of target that can be detected in at least 95% of replicates. The final quadruplex assay resulted to be able to detect at least $1x10^3$ copies/mL of inactivated virus diluted in PBS in 95% of cases for both influenza and RSV targets.

Importantly, we observed that our influenza A, B and RSV assay was able to detect many different common circulating viral strains, from geographically

diverse location. In details, 35 influenza A, 25 influenza B and 11 RSV viruses quantified and titered were successfully detected by the assay.

We also verified the product's ability to exclusively identify influenza A, B and RSV in NPS specimens that contain other closely related microorganisms or viruses. To this aim, we selected cross-reactants that may exhibit similar clinical symptoms as influenza and/or RSV or may be present as normal flora in the NPS and we verified that none of them was amplified by our assay.

Finally, the clinical value of influenza A, B and RSV RT Q-LAMP assay was assessed performing a validation on a total of 120 clinical samples from human patients with signs and symptoms of respiratory tract infections, assayed with standard diagnostic RT-PCR methods routinely used in the hospital laboratory. The results showed that RT Q-LAMP assay has 100% clinical specificity for all the targets assayed and it has a clinical sensitivity ranging from 87% to 90%. Moreover, The Positive Predictive Value (PPV) is 100% while the Negative Predictive Value (NPV) ranges from 96% to 97%.

The peculiar rapidity, simplicity and reliability of this influenza A, B and RSV RT Q-LAMP assay makes it perfect for the fast molecular diagnosis of these infections, allowing clinicians to quickly obtain a results and subsequently treat the patient in the most appropriate way.

The main advantage of the developed assay is that it is extremely rapid, with a time-to-result of only 60 minutes; this time could even be shorter if we consider that the positive samples with at least $1x10^4$ copies/mL of viruses amplified in less than 40 minutes. Only samples with $1x10^3$ copies/mL or less viruses gave amplification results between 40 and 60 minutes. Considering that diagnosis specimens are frequently characterized by a viral load above

$1x10^4$ copies/mL, we can conclude that our RT Q-LAMP assay could be able to provide a diagnosis in less than 40 minutes.

Considering that DAD and Liaison MDX can analyze eight samples at a time, assay use is extremely advantageous in all those contexts in which clinicians require answers for a limited number of samples in a short time. On the contrary, laboratory routine screening activities involve tens or even hundreds of samples per day, usually analyzable with high-throughput platforms, which however require many hours of processing and analysis before providing a diagnosis. Therefore, RT Q-LAMP assay could be extremely appropriate and suitable in clinical urgencies when it is necessary to test a limited number of samples, giving the result in the shortest possible time.

Importantly, Liaison MDX instrument is small, handy and easily transportable. Thus, our influenza A, B and RSV RT Q-LAMP assay can be easily used to test patients with suspected symptoms in extra-hospital triage structures or in vans, before entering the hospital emergency room, avoiding possible subsequent nosocomial outbreaks.

Finally, it is likely that recent COVID-19 pandemic completely changes viral molecular diagnostic scenario: in fact, before 2019, the majority of influenza diagnosis were made mostly on the basis of clinical symptoms. Nowadays, considering that influenza and COVID-19 symptoms are similar, it could be hard to tell the difference between them based on symptoms alone. With this in mind, in the coming months and years, rapid influenza tests, as our RT Q-LAMP assay, could become increasingly critical in determining whether patients with respiratory tract infection are sick with influenza rather than COVID-19.

In the next future, further tests will be fundamental to deeply evaluate the industrialization of this assay so as to have the possibility to launch it on the market as diagnostic product able to quickly detect and discriminate influenza A, B and RSV, with high sensitivity and specificity.

REFERENCES

Abdullah, J., Saffie, N., Sjasri, F. A. R., Husin, A., Abdul-Rahman, Z., Ismail, A., Aziah, I., & Mohamed, M. (2014). Rapid detection of Salmonella Typhi by loop-mediated isothermal amplification (LAMP) method. *Brazilian Journal of Microbiology*, *45*(4), 1385–1391. https://doi.org/10.1590/S1517-83822014000400032

Adao, D. E. V, & Rivera, W. L. (2016). Loop-mediated isothermal amplification (LAMP) assay for the rapid detection of the sexually-transmitted parasite, Trichomonas vaginalis. *Annals of Parasitology*, *62*(1), 25–31. https://doi.org/10.17420/ap6201.28

Agoti, C. N., Mwihuri, A. G., Sande, C. J., Onyango, C. O., Medley, G. F., Cane, P. A., & Nokes, D. J. (2012). Genetic Relatedness of Infecting and Reinfecting Respiratory Syncytial Virus Strains Identified in a Birth Cohort From Rural Kenya. *The Journal of Infectious Diseases*, *206*(10), 1532–1541. https://doi.org/10.1093/infdis/jis570

Agoti, C. N., Otieno, J. R., Gitahi, C. W., Cane, P. A., & Nokes, D. J. (2014). Rapid Spread and Diversification of Respiratory Syncytial Virus Genotype ON1, Kenya. *Emerging Infectious Diseases*, *20*(6), 950–959. https://doi.org/10.3201/eid2006.131438

Agoti, C. N., Otieno, J. R., Munywoki, P. K., Mwihuri, A. G., Cane, P. A., Nokes, D. J., Kellam, P., & Cotten, M. (2015). Local Evolutionary Patterns of Human Respiratory Syncytial Virus Derived from Whole-Genome Sequencing. *Journal of Virology*, *89*(7), 3444–3454. https://doi.org/10.1128/JVI.03391-14

Ahn, S. J., Baek, Y. H., Lloren, K. K. S., Choi, W. S., Jeong, J. H., Antigua, K. J. C.,

Kwon, H. Il, Park, S. J., Kim, E. H., Kim, Y. Il, Si, Y. J., Hong, S. B., Shin, K. S., Chun, S., Choi, Y. K., & Song, M. S. (2019). Rapid and simple colorimetric detection of multiple influenza viruses infecting humans using a reverse transcriptional loop-mediated isothermal amplification (RT-LAMP) diagnostic platform. *BMC Infectious Diseases*, *19*(1), 1–12. https://doi.org/10.1186/s12879-019-4277-8

Al-Toum, R., Bdour, S., & Ayyash, H. (2006). Epidemiology and Clinical Characteristics of Respiratory Syncytial Virus Infections in Jordan. *Journal of Tropical Pediatrics*, *52*(4), 282–287. https://doi.org/10.1093/tropej/fml002

Aryan, E., Makvandi, M., Farajzadeh, A., Huygen, K., Bifani, P., Mousavi, S. L., Fateh, A., Jelodar, A., Gouya, M. M., & Romano, M. (2010). A novel and more sensitive loop-mediated isothermal amplification assay targeting IS6110 for detection of Mycobacterium tuberculosis complex. *Microbiological Research*, *165*(3), 211–220. https://doi.org/10.1016/j.micres.2009.05.001

Auksornkitti, V., Kamprasert, N., Thongkomplew, S., Suwannakarn, K., Theamboonlers, A., Samransamruajkij, R., & Poovorawan, Y. (2014). Molecular characterization of human respiratory syncytial virus, 2010-2011: identification of genotype ON1 and a new subgroup B genotype in Thailand. *Archives of Virology*, *159*(3), 499–507. https://doi.org/10.1007/s00705-013-1773-9

Babu, U. S., Harrison, L. M., Mammel, M. K., Bigley, E. C., Hiett, K. L., & Balan, K. V. (2020). A loop-mediated isothermal amplification (LAMP) assay for the consensus detection of human pathogenic Campylobacter species.

Journal of Microbiological Methods, *176*, 106009. https://doi.org/10.1016/j.mimet.2020.106009

Baek, Y. H., Choi, E. H., Song, M.-S., Pascua, P. N. Q., Kwon, H., Park, S.-J., Lee, J. H., Woo, S.-I., Ahn, B.-H., Han, H.-S., Hahn, Y.-S., Shin, K.-S., Jang, H.-L., Kim, S.-Y., & Choi, Y.-K. (2012). Prevalence and genetic characterization of respiratory syncytial virus (RSV) in hospitalized children in Korea. *Archives of Virology*, *157*(6), 1039–1050. https://doi.org/10.1007/s00705-012-1267-1

Bahl, J., Nelson, M. I., Chan, K. H., Chen, R., Vijaykrishna, D., Halpin, R. A., Stockwell, T. B., Lin, X., Wentworth, D. E., Ghedin, E., Guan, Y., Peiris, J. S. M., Riley, S., Rambaut, A., Holmes, E. C., & Smith, G. J. D. (2011). Temporally structured metapopulation dynamics and persistence of influenza A H3N2 virus in humans. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(48), 19359–19364. https://doi.org/10.1073/pnas.1109314108

Baillie, G. J., Galiano, M., Agapow, P.-M., Myers, R., Chiam, R., Gall, A., Palser, A. L., Watson, S. J., Hedge, J., Underwood, A., Platt, S., McLean, E., Pebody, R. G., Rambaut, A., Green, J., Daniels, R., Pybus, O. G., Kellam, P., & Zambon, M. (2012). Evolutionary Dynamics of Local Pandemic H1N1/2009 Influenza Virus Lineages Revealed by Whole-Genome Analysis. *Journal of Virology*, *86*(1), 11–18. https://doi.org/10.1128/JVI.05347-11

Bakhtiari, S., Alvandi, A., Pajavand, H., Navabi, J., Najafi, F., & Abiri, R. (2016). Development and Diagnostic Evaluation of Loop-Mediated Isothermal Amplification Using a New Gene Target for Rapid Detection of

Helicobacter pylori. *Jundishapur Journal of Microbiology*, *9*(5). https://doi.org/10.5812/jjm.28831

Barenfanger, J., Drake, C., Mueller, T., Troutt, T., O'Brien, J., & Guttman, K. (2001). R-Mix cells are faster, at least as sensitive and marginally more costly than conventional cell lines for the detection of respiratory viruses. *Journal of Clinical Virology*, *22*(1), 101–110. https://doi.org/10.1016/S1386-6532(01)00171-8

Beckhaus, A. A., & Castro-Rodriguez, J. A. (2018). Down Syndrome and the Risk of Severe RSV Infection: A Meta-analysis. *Pediatrics*, *142*(3), e20180225. https://doi.org/10.1542/peds.2018-0225

Bell, J. J., Anderson, E. J., Greene, W. H., Romero, J. R., Merchant, M., & Selvarangan, R. (2014). Multicenter clinical performance evaluation of BD Veritor™ System for Rapid Detection of Respiratory Syncytial Virus. *Journal of Clinical Virology*, *61*(1), 113–117. https://doi.org/10.1016/j.jcv.2014.05.020

Bennett, J., Dolin, R., & Blaser, M. (2015). *Mandell, Douglas, and Bennett's principles and practices of infectious diseases* (8th editio). Elsevier.

Bentaleb, E. M., Abid, M., El Messaoudi, M. D., Lakssir, B., Ressami, E. M., Amzazi, S., Sefrioui, H., & Ait Benhassou, H. (2016). Development and evaluation of an in-house single step loop-mediated isothermal amplification (SS-LAMP) assay for the detection of Mycobacterium tuberculosis complex in sputum samples from Moroccan patients. *BMC Infectious Diseases*, *16*(1), 517. https://doi.org/10.1186/s12879-016-1864-9

Bentzen, E. L., House, F., Utley, T. J., Crowe, J. E., & Wright, D. W. (2005).

Progression of Respiratory Syncytial Virus Infection Monitored by Fluorescent Quantum Dot Probes. *Nano Letters*, *5*(4), 591–595. https://doi.org/10.1021/nl048073u

Bermingham, A., & Collins, P. L. (1999). The M2-2 protein of human respiratory syncytial virus is a regulatory factor involved in the balance between RNA replication and transcription. *Proceedings of the National Academy of Sciences*, *96*(20), 11259–11264. https://doi.org/10.1073/pnas.96.20.11259

Bhadra, S., Jiang, Y. S., Kumar, M. R., Johnson, R. F., Hensley, L. E., & Ellington, A. D. (2015). Real-time sequence-validated loop-mediated isothermal amplification assays for detection of Middle East respiratory syndrome coronavirus (MERS-CoV). *PloS One*, *10*(4), e0123126. https://doi.org/10.1371/journal.pone.0123126

Bhandary, R. (2016). Detection of Respiratory Syncytial Virus using Direct Fluorescent Antibody Assay in Paediatric Patients with Acute Respiratory Tract Infection. *JOURNAL OF CLINICAL AND DIAGNOSTIC RESEARCH*. https://doi.org/10.7860/JCDR/2016/17530.8044

Biere, B., Bauer, B., & Schweiger, B. (2010). Differentiation of influenza b virus lineages yamagata and victoria by real-time PCR. *Journal of Clinical Microbiology*, *48*(4), 1425–1427. https://doi.org/10.1128/JCM.02116-09

Binder, W., Thorsen, J., & Borczuk, P. (2017). RSV in adult ED patients: Do emergency providers consider RSV as an admission diagnosis? *The American Journal of Emergency Medicine*, *35*(8), 1162–1165. https://doi.org/10.1016/j.ajem.2017.06.022

Bonney, L. C., Watson, R. J., Slack, G. S., Bosworth, A., Wand, N. I. V., &

Hewson, R. (2020). A flexible format LAMP assay for rapid detection of Ebola virus. *PLOS Neglected Tropical Diseases*, *14*(7), e0008496. https://doi.org/10.1371/journal.pntd.0008496

Borek, A. P., Clemens, S. H., Gaskins, V. K., Aird, D. Z., & Valsamakis, A. (2006). Respiratory Syncytial Virus Detection by Remel Xpect, Binax Now RSV, Direct Immunofluorescent Staining, and Tissue Culture. *Journal of Clinical Microbiology*, *44*(3), 1105–1107. https://doi.org/10.1128/JCM.44.3.1105-1107.2006

Bouvier, N. M., & Palese, P. (2008). The biology of influenza viruses. *Vaccine*, *26*(SUPPL. 4), 49–53. https://doi.org/10.1016/j.vaccine.2008.07.039

Briedis, D. J., & Lamb, R. A. (1982). Influenza B virus genome: sequences and structural organization of RNA segment 8 and the mRNAs coding for the NS1 and NS2 proteins. *Journal of Virology*, *42*(1), 186–193. https://doi.org/10.1128/jvi.42.1.186-193.1982

Briedis, D. J., Lamb, R. A., & Choppin, P. W. (1982). Sequence of RNA segment 7 of the influenza B virus genome: partial amino acid homology between the membrane proteins (M1) of influenza A and B viruses and conservation of a second open reading frame. *Virology*, *116*(2), 581–588.

Britton, S., Cheng, Q., Grigg, M. J., William, T., Anstey, N. M., & McCarthy, J. S. (2016). A Sensitive, Colorimetric, High-Throughput Loop-Mediated Isothermal Amplification Assay for the Detection of Plasmodium knowlesi. *The American Journal of Tropical Medicine and Hygiene*, *95*(1), 120–122. https://doi.org/10.4269/ajtmh.15-0670

Brock, S. C., Goldenring, J. R., & Crowe, J. E. (2003). Apical recycling systems regulate directional budding of respiratory syncytial virus from polarized

epithelial cells. *Proceedings of the National Academy of Sciences*, *100*(25), 15143–15148. https://doi.org/10.1073/pnas.2434327100

Bruning, A. H. L., Leeflang, M. M. G., Vos, J. M. B. W., Spijker, R., de Jong, M. D., Wolthers, K. C., & Pajkrt, D. (2017). Rapid Tests for Influenza, Respiratory Syncytial Virus, and Other Respiratory Viruses: A Systematic Review and Meta-analysis. *Clinical Infectious Diseases*, *65*(6), 1026–1032. https://doi.org/10.1093/cid/cix461

Cai, T., Lou, G. Q., Yang, J., Xu, D., & Meng, Z. H. (2008). Development and evaluation of real-time loop-mediated isothermal amplification for hepatitis B virus DNA quantification: A new tool for HBV management. *Journal of Clinical Virology*, *41*(4), 270–276. https://doi.org/10.1016/j.jcv.2007.11.025

Cane, P. A., Matthews, D. A., & Pringle, C. R. (1994). Analysis of respiratory syncytial virus strain variation in successive epidemics in one city. *Journal of Clinical Microbiology*, *32*(1), 1–4. https://doi.org/10.1128/JCM.32.1.1-4.1994

Centers for Disease Control and Prevention (CDC). (2019). *Antigenic Characterization*.

Centers for Disease Control and Prevention (CDC). (2020a). *RSV Clinical Description and Diagnosis*.

Centers for Disease Control and Prevention (CDC). (2020b). *RSV Seasonal Trends*.

Centers for Disease Control and Prevention (CDC). (2020c). *Types of Influenza Viruses*. https://www.cdc.gov/flu/about/viruses/types.htm

Chartrand, C., Tremblay, N., Renaud, C., & Papenburg, J. (2015). Diagnostic

Accuracy of Rapid Antigen Detection Tests for Respiratory Syncytial Virus Infection: Systematic Review and Meta-analysis. *Journal of Clinical Microbiology*, *53*(12), 3738–3749. https://doi.org/10.1128/JCM.01816-15

Chatzis, O., Darbre, S., Pasquier, J., Meylan, P., Manuel, O., Aubert, J. D., Beck-Popovic, M., Masouridi-Levrat, S., Ansari, M., Kaiser, L., Posfay-Barbe, K. M., & Asner, S. A. (2018). Burden of severe RSV disease among immunocompromised children and adults: a 10 year retrospective study. *BMC Infectious Diseases*, *18*(1), 111. https://doi.org/10.1186/s12879-018-3002-3

Chen, J.-H., Lu, F., Lim, C. S., Kim, J.-Y., Ahn, H.-J., Suh, I.-B., Takeo, S., Tsuboi, T., Sattabongkot, J., & Han, E.-T. (2010). Detection of Plasmodium vivax infection in the Republic of Korea by loop-mediated isothermal amplification (LAMP). *Acta Tropica*, *113*(1), 61–65. https://doi.org/10.1016/j.actatropica.2009.09.007

Chen, W., Calvo, P. A., Malide, D., Gibbs, J., Schubert, U., Bacik, I., Basta, S., O'Neill, R., Schickli, J., Palese, P., Henklein, P., Bennink, J. R., & Yewdell, J. W. (2001). A novel influenza A virus mitochondrial protein that induces cell death. *Nature Medicine*, *7*(12), 1306–1312. https://doi.org/10.1038/nm1201-1306

Chiu, C. Y., Urisman, A., Greenhow, T. L., Rouskin, S., Yagi, S., Schnurr, D., Wright, C., Drew, W. L., Wang, D., Weintrub, P. S., DeRisi, J. L., & Ganem, D. (2008). Utility of DNA Microarrays for Detection of Viruses in Acute Respiratory Tract Infections in Children. *The Journal of Pediatrics*, *153*(1), 76-83.e1. https://doi.org/10.1016/j.jpeds.2007.12.035

Cho, C. H., Lee, C. K., Nam, M.-H., Yoon, S.-Y., Lim, C. S., Cho, Y., & Kim, Y. K. (2014). Evaluation of the AdvanSure™ real-time RT-PCR compared with culture and Seeplex RV15 for simultaneous detection of respiratory viruses. *Diagnostic Microbiology and Infectious Disease*, *79*(1), 14–18. https://doi.org/10.1016/j.diagmicrobio.2014.01.016

Choudhuri, J. A. (2006). Effect of Altitude on Hospitalizations for Respiratory Syncytial Virus Infection. *PEDIATRICS*, *117*(2), 349–356. https://doi.org/10.1542/peds.2004-2795

Collins, P. L., Fearns, R., & Graham, B. S. (2013). *Respiratory Syncytial Virus: Virology, Reverse Genetics, and Pathogenesis of Disease* (pp. 3–38). https://doi.org/10.1007/978-3-642-38919-1_1

Colman, P. M., & Lawrence, M. C. (2003). The structural biology of type I viral membrane fusion. *Nature Reviews Molecular Cell Biology*, *4*(4), 309–319. https://doi.org/10.1038/nrm1076

Colosia, A. D., Yang, J., Hillson, E., Mauskopf, J., Copley-Merriman, C., Shinde, V., & Stoddard, J. (2017). The epidemiology of medically attended respiratory syncytial virus in older adults in the United States: A systematic review. *PLOS ONE*, *12*(8), e0182321. https://doi.org/10.1371/journal.pone.0182321

Cox, N. J., & Subbarao, K. (2000). Global Epidemiology of Influenza: Past and Present. *Annual Review of Medicine*, *51*(1), 407–421. https://doi.org/10.1146/annurev.med.51.1.407

Cruz, A. T., Demmler-Harrison, G. J., Caviness, A. C., Buffone, G. J., & Revell, P. A. (2010). Performance of a Rapid Influenza Test in Children During the H1N1 2009 Influenza A Outbreak. *PEDIATRICS*, *125*(3), e645–e650.

https://doi.org/10.1542/peds.2009-3060

Cullen, L. M., Schmidt, M. R., & Morrison, T. G. (2017). The importance of RSV F protein conformation in VLPs in stimulation of neutralizing antibody titers in mice previously infected with RSV. *Human Vaccines & Immunotherapeutics*, *13*(12), 2814–2823. https://doi.org/10.1080/21645515.2017.1329069

Curtis, K. A., Morrison, D., Rudolph, D. L., Shankar, A., Bloomfield, L. S. P., Switzer, W. M., & Owen, S. M. (2018). A multiplexed RT-LAMP assay for detection of group M HIV-1 in plasma or whole blood. *Journal of Virological Methods*, *255*, 91–97. https://doi.org/10.1016/j.jviromet.2018.02.012

Curtis, K. A., Rudolph, D. L., & Owen, S. M. (2009). Sequence-specific detection method for reverse transcription, loop-mediated isothermal amplification of HIV-1. *Journal of Medical Virology*, *81*(6), 966–972. https://doi.org/10.1002/jmv.21490

Dao Thi, V. L., Herbst, K., Boerner, K., Meurer, M., Kremer, L. P., Kirrmaier, D., Freistaedter, A., Papagiannidis, D., Galmozzi, C., Stanifer, M. L., Boulant, S., Klein, S., Chlanda, P., Khalid, D., Miranda, I. B., Schnitzler, P., Kräusslich, H.-G., Knop, M., & Anders, S. (2020). A colorimetric RT-LAMP assay and LAMP-sequencing for detecting SARS-CoV-2 RNA in clinical samples. *Science Translational Medicine*, eabc7075. https://doi.org/10.1126/scitranslmed.abc7075

Dapat, I. C., Shobugawa, Y., Sano, Y., Saito, R., Sasaki, A., Suzuki, Y., Kumaki, A., Zaraket, H., Dapat, C., Oguma, T., Yamaguchi, M., & Suzuki, H. (2010). New Genotypes within Respiratory Syncytial Virus Group B Genotype BA

in Niigata, Japan. *Journal of Clinical Microbiology*, *48*(9), 3423–3427. https://doi.org/10.1128/JCM.00646-10

Dauber, B., Heins, G., & Wolff, T. (2004). The Influenza B Virus Nonstructural NS1 Protein Is Essential for Efficient Viral Growth and Antagonizes Beta Interferon Induction. *Journal of Virology*, *78*(4), 1865–1872. https://doi.org/10.1128/jvi.78.4.1865-1872.2004

Dawson, E. D., Moore, C. L., Dankbar, D. M., Mehlmann, M., Townsend, M. B., Smagala, J. A., Smith, C. B., Cox, N. J., Kuchta, R. D., & Rowlen, K. L. (2007). Identification of A/H5N1 Influenza Viruses Using a Single Gene Diagnostic Microarray. *Analytical Chemistry*, *79*(1), 378–384. https://doi.org/10.1021/ac061920o

de-Paris, F., Beck, C., de Souza Nunes, L., Pinheiro, A. M., Paiva, R. M., da Silva Menezes, D., Pires, M. R., dos Santos, R. P., de Souza Kuchenbecker, R., & Barth, A. L. (2014). Evaluation of respiratory syncytial virus group A and B genotypes among nosocomial and community-acquired pediatric infections in southern Brazil. *Virology Journal*, *11*(1), 36. https://doi.org/10.1186/1743-422X-11-36

Dessilly, G., Goeminne, L., Vandenbroucke, A., Dufrasne, F. E., Martin, A., & Kabamba-Mukabi, B. (2018). First evaluation of the Next-Generation Sequencing platform for the detection of HIV-1 drug resistance mutations in Belgium. *PLOS ONE*, *13*(12), e0209561. https://doi.org/10.1371/journal.pone.0209561

DeVincenzo, J. P., McClure, M. W., Symons, J. A., Fathi, H., Westland, C., Chanda, S., Lambkin-Williams, R., Smith, P., Zhang, Q., Beigelman, L., Blatt, L. M., & Fry, J. (2015). Activity of Oral ALS-008176 in a Respiratory

Syncytial Virus Challenge Study. *New England Journal of Medicine*, *373*(21), 2048–2058. https://doi.org/10.1056/NEJMoa1413275

Doan, Q., Enarson, P., Kissoon, N., Klassen, T. P., & Johnson, D. W. (2014). Rapid viral diagnosis for acute febrile respiratory illness in children in the Emergency Department. *Cochrane Database of Systematic Reviews*. https://doi.org/10.1002/14651858.CD006452.pub4

Drexler, J. F., Helmer, A., Kirberg, H., Reber, U., Panning, M., Müller, M., Höfling, K., Matz, B., Drosten, C., & Eis-Hübinger, A. M. (2009). Poor Clinical Sensitivity of Rapid Antigen Test for Influenza A Pandemic (H1N1) 2009 Virus. *Emerging Infectious Diseases*, *15*(10), 1662–1664. https://doi.org/10.3201/eid1510.091186

Dunning, J., Thwaites, R. S., & Openshaw, P. J. M. (2020). Seasonal and pandemic influenza: 100 years of progress, still much to learn. *Mucosal Immunology*, *13*(4), 566–573. https://doi.org/10.1038/s41385-020-0287-5

Espinosa, Y., San Martín, C., Torres, A., Farfán, M., Torres, J., Avadhanula, V., Piedra, P., & Tapia, L. (2017). Genomic Loads and Genotypes of Respiratory Syncytial Virus: Viral Factors during Lower Respiratory Tract Infection in Chilean Hospitalized Infants. *International Journal of Molecular Sciences*, *18*(3), 654. https://doi.org/10.3390/ijms18030654

Fallahi, S., Babaei, M., Rostami, A., Mirahmadi, H., Arab-Mazar, Z., & Sepahvand, A. (2020). Diagnosis of Candida albicans: conventional diagnostic methods compared to the loop-mediated isothermal amplification (LAMP) assay. *Archives of Microbiology*, *202*(2), 275–282. https://doi.org/10.1007/s00203-019-01736-7

Falsey, A. R., Hennessey, P. A., Formica, M. A., Cox, C., & Walsh, E. E. (2005). Respiratory Syncytial Virus Infection in Elderly and High-Risk Adults. *New England Journal of Medicine*, *352*(17), 1749–1759. https://doi.org/10.1056/NEJMoa043951

Falsey, A. R., McElhaney, J. E., Beran, J., van Essen, G. A., Duval, X., Esen, M., Galtier, F., Gervais, P., Hwang, S.-J., Kremsner, P., Launay, O., Leroux-Roels, G., McNeil, S. A., Nowakowski, A., Richardus, J. H., Ruiz-Palacios, G., St Rose, S., Devaster, J.-M., Oostvogels, L., … Taylor, S. (2014). Respiratory Syncytial Virus and Other Respiratory Viral Infections in Older Adults With Moderate to Severe Influenza-like Illness. *The Journal of Infectious Diseases*, *209*(12), 1873–1881. https://doi.org/10.1093/infdis/jit839

Fan, F., Du, P., Kan, B., & Yan, M. (2015). The Development and Evaluation of a Loop-Mediated Isothermal Amplification Method for the Rapid Detection of Salmonella enterica serovar Typhi. *PLOS ONE*, *10*(4), e0124507. https://doi.org/10.1371/journal.pone.0124507

Fan, S.-J., Tan, H.-K., Xu, Y.-C., Chen, Y.-Z., Xie, T.-A., Pan, Z.-Y., Ouyang, S., Li, Q., Li, X., Li, Z.-X., & Guo, X.-G. (2020). A pooled analysis of the LAMP assay for the detection of Neisseria meningitidis. *BMC Infectious Diseases*, *20*(1), 525. https://doi.org/10.1186/s12879-020-05250-w

Fearns, R., Collins, P. L., & Peeples, M. E. (2000). Functional Analysis of the Genomic and Antigenomic Promoters of Human Respiratory Syncytial Virus. *Journal of Virology*, *74*(13), 6006–6014. https://doi.org/10.1128/JVI.74.13.6006-6014.2000

Fearns, R., Peeples, M. E., & Collins, P. L. (2002). Mapping the Transcription

and Replication Promoters of Respiratory Syncytial Virus. *Journal of Virology*, *76*(4), 1663–1672. https://doi.org/10.1128/JVI.76.4.1663-1672.2002

Foo, P. C., Chan, Y. Y., Mohamed, M., Wong, W. K., Nurul Najian, A. B., & Lim, B. H. (2017). Development of a thermostabilised triplex LAMP assay with dry-reagent four target lateral flow dipstick for detection of Entamoeba histolytica and non-pathogenic Entamoeba spp. *Analytica Chimica Acta*, *966*, 71–80. https://doi.org/10.1016/j.aca.2017.02.019

Förster, A., Maertens, G. N., Farrell, P. J., & Bajorek, M. (2015). Dimerization of Matrix Protein Is Required for Budding of Respiratory Syncytial Virus. *Journal of Virology*, *89*(8), 4624–4635. https://doi.org/10.1128/JVI.03500-14

Francois, P., Tangomo, M., Hibbs, J., Bonetti, E.-J., Boehme, C. C., Notomi, T., Perkins, M. D., & Schrenzel, J. (2011). Robustness of a loop-mediated isothermal amplification reaction for diagnostic applications. *FEMS Immunology & Medical Microbiology*, *62*(1), 41–48. https://doi.org/10.1111/j.1574-695X.2011.00785.x

Fricke, J., Koo, L. Y., Brown, C. R., & Collins, P. L. (2013). p38 and OGT Sequestration into Viral Inclusion Bodies in Cells Infected with Human Respiratory Syncytial Virus Suppresses MK2 Activities and Stress Granule Assembly. *Journal of Virology*, *87*(3), 1333–1347. https://doi.org/10.1128/JVI.02263-12

Fujino, M., Suzuki, E., Watanabe, M., & Nakayama, T. (2015). Loop-Mediated Isothermal Amplification (LAMP) Aids the Clinical Diagnosis of Pertussis. *Japanese Journal of Infectious Diseases*, *68*(6), 532–533.

https://doi.org/10.7883/yoken.JJID.2015.125

Fukuta, S., Iida, T., Mizukami, Y., Ishida, A., Ueda, J., Kanbe, M., & Ishimoto, Y. (2003). Detection of Japanese yam mosaic virus by RT-LAMP. *Archives of Virology*, *148*(9), 1713–1720. https://doi.org/10.1007/s00705-003-0134-5

Gaitonde, D. Y., Moore, F. C., & Morgan, M. K. (2019). Influenza: Diagnosis and Treatment. *American Family Phisician*, *Dec 15*(100(12)), 751–758.

Gall, A., Hoffmann, B., Harder, T., Grund, C., Hoper, D., & Beer, M. (2009). Design and Validation of a Microarray for Detection, Hemagglutinin Subtyping, and Pathotyping of Avian Influenza Viruses. *Journal of Clinical Microbiology*, *47*(2), 327–334. https://doi.org/10.1128/JCM.01330-08

Gan, S. W., Ng, L., Lin, X., Gong, X., & Torres, J. (2008). Structure and ion channel activity of the human respiratory syncytial virus (hRSV) small hydrophobic protein transmembrane domain. *Protein Science*, *17*(5), 813–820. https://doi.org/10.1110/ps.073366208

García-Barreno, B., Delgado, T., & Melero, J. A. (1996). Identification of protein regions involved in the interaction of human respiratory syncytial virus phosphoprotein and nucleoprotein: significance for nucleocapsid assembly and formation of cytoplasmic inclusions. *Journal of Virology*, *70*(2), 801–808. https://doi.org/10.1128/JVI.70.2.801-808.1996

García-Sastre, A. (2001). Inhibition of interferon-mediated antiviral responses by influenza A viruses and other negative-strand RNA viruses. *Virology*, *279*(2), 375–384. https://doi.org/10.1006/viro.2000.0756

García, J., García-Barreno, B., Vivo, A., & Melero, J. A. (1993). Cytoplasmic

Inclusions of Respiratory Syncytial Virus-Infected Cells: Formation of Inclusion Bodies in Transfected Cells That Coexpress the Nucleoprotein, the Phosphoprotein, and the 22K Protein. *Virology*, *195*(1), 243–247. https://doi.org/10.1006/viro.1993.1366

Ge, Y., Cui, L., Qi, X., Shan, J., Shan, Y., Qi, Y., Wu, B., Wang, H., & Shi, Z. (2010). Detection of novel swine origin influenza A virus (H1N1) by real-time nucleic acid sequence-based amplification. *Journal of Virological Methods*, *163*(2), 495–497. https://doi.org/10.1016/j.jviromet.2009.10.025

Ginocchio, C. C. (2007). Detection of respiratory viruses using non-molecular based methods. *Journal of Clinical Virology*, *40*, S11–S14. https://doi.org/10.1016/S1386-6532(07)70004-5

Gower, T. L., Pastey, M. K., Peeples, M. E., Collins, P. L., McCurdy, L. H., Hart, T. K., Guth, A., Johnson, T. R., & Graham, B. S. (2005). RhoA Signaling Is Required for Respiratory Syncytial Virus-Induced Syncytium Formation and Filamentous Virion Morphology. *Journal of Virology*, *79*(9), 5326–5336. https://doi.org/10.1128/JVI.79.9.5326-5336.2005

Grad, Y. H., Newman, R., Zody, M., Yang, X., Murphy, R., Qu, J., Malboeuf, C. M., Levin, J. Z., Lipsitch, M., & DeVincenzo, J. (2014). Within-Host Whole-Genome Deep Sequencing and Diversity Analysis of Human Respiratory Syncytial Virus Infection Reveals Dynamics of Genomic Diversity in the Absence and Presence of Immune Pressure. *Journal of Virology*, *88*(13), 7286–7293. https://doi.org/10.1128/JVI.00038-14

Greninger, A. L., Chen, E. C., Sittler, T., Scheinerman, A., Roubinian, N., Yu, G., Kim, E., Pillai, D. R., Guyard, C., Mazzulli, T., Isa, P., Arias, C. F., Hackett,

J., Schochetman, G., Miller, S., Tang, P., & Chiu, C. Y. (2010). A Metagenomic Analysis of Pandemic Influenza A (2009 H1N1) Infection in Patients from North America. *PLoS ONE*, *5*(10), e13381. https://doi.org/10.1371/journal.pone.0013381

Griffiths, C., Drews, S. J., & Marchant, D. J. (2017). Respiratory Syncytial Virus: Infection, Detection, and New Options for Prevention and Treatment. *Clinical Microbiology Reviews*, *30*(1), 277–319. https://doi.org/10.1128/CMR.00010-16

Hall, C. B. (2001). Respiratory Syncytial Virus and Parainfluenza Virus. *New England Journal of Medicine*, *344*(25), 1917–1928. https://doi.org/10.1056/NEJM200106213442507

Hancock, K., Veguilla, V., Lu, X., Zhong, W., Butler, E. N., Sun, H., Liu, F., Dong, L., DeVos, J. R., Gargiullo, P. M., Brammer, T. L., Cox, N. J., Tumpey, T. M., & Katz, J. M. (2009). Cross-Reactive Antibody Responses to the 2009 Pandemic H1N1 Influenza Virus. *New England Journal of Medicine*, *361*(20), 1945–1952. https://doi.org/10.1056/NEJMoa0906453

Harper, S. A., Bradley, J. S., Englund, J. A., File, T. M., Gravenstein, S., Hayden, F. G., McGeer, A. J., Neuzil, K. M., Pavia, A. T., Tapper, M. L., Uyeki, T. M., & Zimmerman, R. K. (2009). Seasonal Influenza in Adults and Children—Diagnosis, Treatment, Chemoprophylaxis, and Institutional Outbreak Management: Clinical Practice Guidelines of the Infectious Diseases Society of America. *Clinical Infectious Diseases*, *48*(8), 1003–1032. https://doi.org/10.1086/598513

Hatta, M., & Kawaoka, Y. (2003). The NB Protein of Influenza B Virus Is Not Necessary for Virus Replication In Vitro. *Journal of Virology*, *77*(10),

6050–6054. https://doi.org/10.1128/jvi.77.10.6050-6054.2003

Hawkinson, D., Abhyankar, S., Aljitawi, O., Ganguly, S., McGuirk, J. P., & Horvat, R. (2013). Delayed RSV diagnosis in a stem cell transplant population due to mutations that result in negative polymerase chain reaction. *Diagnostic Microbiology and Infectious Disease*, *75*(4), 426–430. https://doi.org/10.1016/j.diagmicrobio.2012.12.014

Hayden, R. T., Wick, M. T., Rodriguez, A. B., Caliendo, A. M., Mitchell, M. J., & Ginocchio, C. C. (2010). A survey-based assessment of United States clinical laboratory response to the 2009 H1N1 influenza outbreak. *Archives of Pathology & Laboratory Medicine*, *134*(11), 1671–1678. https://doi.org/10.1043/2010-0134-CPR.1

Henderson, G., Murray, J., & Yeo, R. P. (2002). Sorting of the Respiratory Syncytial Virus Matrix Protein into Detergent-Resistant Structures Is Dependent on Cell-Surface Expression of the Glycoproteins. *Virology*, *300*(2), 244–254. https://doi.org/10.1006/viro.2002.1540

Hendry, R. M., Talis, A. L., Godfrey, E., Anderson, L. J., Fernie, B. F., & McIntosh, K. (1986). Concurrent Circulation of Antigenically Distinct Strains of Respiratory Syncytial Virus During Community Outbreaks. *Journal of Infectious Diseases*, *153*(2), 291–297. https://doi.org/10.1093/infdis/153.2.291

Higashimoto, Y., Kawamura, Y., Kuboshiki, A., Hattori, F., Miura, H., Nishimura, N., Ozaki, T., Ihira, M., & Yoshikawa, T. (2019). Reliability of direct varicella zoster virus loop-mediated isothermal amplification method for rapid diagnosis of breakthrough varicella. *Journal of Clinical Virology*, *119*, 53–58. https://doi.org/10.1016/j.jcv.2019.07.009

Hoos, J., Peters, R. M., Tabatabai, J., Grulich-Henn, J., Schnitzler, P., & Pfeil, J. (2017). Reverse-transcription loop-mediated isothermal amplification for rapid detection of respiratory syncytial virus directly from nasopharyngeal swabs. *Journal of Virological Methods*, *242*, 53–57. https://doi.org/10.1016/j.jviromet.2017.01.006

Horiuchi, S., Nakano, R., Nakano, A., Hishiya, N., Uno, K., Suzuki, Y., Tanouchi, A., Kakuta, N., Masui, T., Jojima, N., & Yano, H. (2019). Development of a loop-mediated isothermal amplification assay for rapid Helicobacter pylori detection. *Journal of Microbiological Methods*, *163*, 105653. https://doi.org/10.1016/j.mimet.2019.105653

Horvath, C. M., Williams, M. A., & Lamb, R. A. (1990). Eukaryotic coupled translation of tandem cistrons: Identificaiton of the influenza B virus BM2 polypeptide. *EMBO Journal*, *9*(8), 2639–2647. https://doi.org/10.1002/j.1460-2075.1990.tb07446.x

Hu, A., Colella, M., Tam, J. S., Rappaport, R., & Cheng, S.-M. (2003). Simultaneous Detection, Subgrouping, and Quantitation of Respiratory Syncytial Virus A and B by Real-Time PCR. *Journal of Clinical Microbiology*, *41*(1), 149–154. https://doi.org/10.1128/JCM.41.1.149-154.2003

Hu, S., Li, M., Zhong, L., Lu, S., Liu, Z., Pu, J., Wen, J., & Huang, X. (2015). Development of reverse-transcription loop-mediated isothermal amplification assay for rapid detection and differentiation of dengue virus serotypes 1–4. *BMC Microbiology*, *15*(1), 265. https://doi.org/10.1186/s12866-015-0595-1

Hu, X., Deng, Q., Li, J., Chen, J., Wang, Z., Zhang, X., Fang, Z., Li, H., Zhao, Y.,

Yu, P., Li, W., Wang, X., Li, S., Zhang, L., & Hou, T. (2020). Development and Clinical Application of a Rapid and Sensitive Loop-Mediated Isothermal Amplification Test for SARS-CoV-2 Infection. *MSphere*, *5*(4). https://doi.org/10.1128/mSphere.00808-20

Hu, Y., Wan, Z., Mu, Y., Zhou, Y., Liu, J., Zhang, C., & Lan, K. (2019). A quite sensitive fluorescent loop-mediated isothermal amplification for rapid detection of respiratory syncytial virus. *Journal of Infection in Developing Countries*, *13*(12), 1135–1141. https://doi.org/10.3855/jidc.11549

Huang, J., Sun, Y., Liu, Y., Xiao, H., & Zhuang, S. (2012). Development of a loop-mediated isothermal amplification method for rapid detection of caprine arthritis-encephalitis virus proviral DNA. *Archives of Virology*, *157*(8), 1463–1469. https://doi.org/10.1007/s00705-012-1322-y

Huang, W. E., Lim, B., Hsu, C. C., Xiong, D., Wu, W., Yu, Y., Jia, H., Wang, Y., Zeng, Y., Ji, M., Chang, H., Zhang, X., Wang, H., & Cui, Z. (2020). RT-LAMP for rapid diagnosis of coronavirus SARS-CoV-2. *Microbial Biotechnology*, *13*(4), 950–961. https://doi.org/10.1111/1751-7915.13586

Imai, K., Tamura, K., Tanigaki, T., Takizawa, M., Nakayama, E., Taniguchi, T., Okamoto, M., Nishiyama, Y., Tarumoto, N., Mitsutake, K., Murakami, T., Maesaki, S., & Maeda, T. (2018). Whole Genome Sequencing of Influenza A and B Viruses With the MinION Sequencer in the Clinical Setting: A Pilot Study. *Frontiers in Microbiology*, *9*. https://doi.org/10.3389/fmicb.2018.02748

Imai, M., Ninomiya, A., Minekawa, H., Notomi, T., Ishizaki, T., Tashiro, M., & Odagiri, T. (2006). Development of H5-RT-LAMP (loop-mediated isothermal amplification) system for rapid diagnosis of H5 avian

influenza virus infection. *Vaccine*, *24*(44–46), 6679–6682. https://doi.org/10.1016/j.vaccine.2006.05.046

Imai, M., Watanabe, T., Hatta, M., Das, S. C., Ozawa, M., Shinya, K., Zhong, G., Hanson, A., Katsura, H., Watanabe, S., Li, C., Kawakami, E., Yamada, S., Kiso, M., Suzuki, Y., Maher, E. A., Neumann, G., & Kawaoka, Y. (2012). Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. *Nature*, *486*(7403), 420–428. https://doi.org/10.1038/nature10831

Ito, M., Watanabe, M., Nakagawa, N., Ihara, T., & Okuno, Y. (2006). Rapid detection and typing of influenza A and B by loop-mediated isothermal amplification: Comparison with immunochromatography and virus isolation. *Journal of Virological Methods*, *135*(2), 272–275. https://doi.org/10.1016/j.jviromet.2006.03.003

Ivey, K. S., Edwards, K. M., & Talbot, H. K. (2018). Respiratory Syncytial Virus and Associations With Cardiovascular Disease in Adults. *Journal of the American College of Cardiology*, *71*(14), 1574–1583. https://doi.org/10.1016/j.jacc.2018.02.013

Jain, S., Self, W. H., Wunderink, R. G., Fakhran, S., Balk, R., Bramley, A. M., Reed, C., Grijalva, C. G., Anderson, E. J., Courtney, D. M., Chappell, J. D., Qi, C., Hart, E. M., Carroll, F., Trabue, C., Donnelly, H. K., Williams, D. J., Zhu, Y., Arnold, S. R., … Finelli, L. (2015). Community-Acquired Pneumonia Requiring Hospitalization among U.S. Adults. *New England Journal of Medicine*, *373*(5), 415–427. https://doi.org/10.1056/NEJMoa1500245

Jeffree, C. E., Rixon, H. W. M. ., Brown, G., Aitken, J., & Sugrue, R. J. (2003).

Distribution of the attachment (G) glycoprotein and GM1 within the envelope of mature respiratory syncytial virus filaments revealed using field emission scanning electron microscopy. *Virology*, *306*(2), 254–267. https://doi.org/10.1016/S0042-6822(02)00016-8

Jerome, H., Taylor, C., Sreenu, V. B., Klymenko, T., Filipe, A. D. S., Jackson, C., Davis, C., Ashraf, S., Wilson-Davies, E., Jesudason, N., Devine, K., Harder, L., Aitken, C., Gunson, R., & Thomson, E. C. (2019). Metagenomic next-generation sequencing aids the diagnosis of viral infections in febrile returning travellers. *Journal of Infection*, *79*(4), 383–388. https://doi.org/10.1016/j.jinf.2019.08.003

Johnson, J. E., Gonzales, R. A., Olson, S. J., Wright, P. F., & Graham, B. S. (2007). The histopathology of fatal untreated human respiratory syncytial virus infection. *Modern Pathology*, *20*(1), 108–119. https://doi.org/10.1038/modpathol.3800725

Johnson, T. R., & Graham, B. S. (2004). Contribution of respiratory syncytial virus G antigenicity to vaccine-enhanced illness and the implications for severe disease during primary respiratory syncytial virus infection. *The Pediatric Infectious Disease Journal*, *23*(Supplement), S46–S57. https://doi.org/10.1097/01.inf.0000108192.94692.d2

Kadmon, G., Levy, I., Mandelboim, M., Nahum, E., Stein, J., Dovrat, S., & Schonfeld, T. (2013). Polymerase-chain-reaction-based diagnosis of viral pulmonary infections in immunocompromised children. *Acta Paediatrica*, *102*(6), e263–e268. https://doi.org/10.1111/apa.12207

Kamachi, K., Moriuchi, T., Hiramatsu, Y., Otsuka, N., & Shibayama, K. (2017). Evaluation of a commercial loop-mediated isothermal amplification

assay for diagnosis of Bordetella pertussis infection. *Journal of Microbiological Methods*, *133*, 20–22. https://doi.org/10.1016/j.mimet.2016.12.009

Kaneko, H., Iida, T., Aoki, K., Ohno, S., & Suzutani, T. (2005). Sensitive and Rapid Detection of Herpes Simplex Virus and Varicella-Zoster Virus. *Journal of Clinical Microbiology*, *43*(7), 3290. https://doi.org/10.1128/JCM.43.7.3290

Kaneko, H., Kawana, T., Fukushima, E., & Suzutani, T. (2007). Tolerance of loop-mediated isothermal amplification to a culture medium and biological substances. *Journal of Biochemical and Biophysical Methods*, *70*(3), 499–501. https://doi.org/10.1016/j.jbbm.2006.08.008

Kanwar, N., Hassan, F., Nguyen, A., & Selvarangan, R. (2015). Head-to-head comparison of the diagnostic accuracies of BD Veritor™ System RSV and Quidel® Sofia® RSV FIA systems for respiratory syncytial virus (RSV) diagnosis. *Journal of Clinical Virology*, *65*, 83–86. https://doi.org/10.1016/j.jcv.2015.02.008

Kashir, J., & Yaqinuddin, A. (2020). Loop mediated isothermal amplification (LAMP) assays as a rapid diagnostic for COVID-19. *Medical Hypotheses*, *141*. https://doi.org/10.1016/j.mehy.2020.109786

Katz, J. M., Hancock, K., & Xu, X. (2011). Serologic assays for influenza surveillance, diagnosis and vaccine evaluation. *Expert Review of Anti-Infective Therapy*, *9*(6), 669–683. https://doi.org/10.1586/eri.11.51

Ke, Z., Dillard, R., Chirkova, T., Leon, F., Stobart, C., Hampton, C., Strauss, J., Rajan, D., Rostad, C., Taylor, J., Yi, H., Shah, R., Jin, M., Hartert, T., Peebles, R., Graham, B., Moore, M., Anderson, L., & Wright, E. (2018).

The Morphology and Assembly of Respiratory Syncytial Virus Revealed by Cryo-Electron Tomography. *Viruses*, *10*(8), 446. https://doi.org/10.3390/v10080446

Khor, C.-S., Sam, I.-C., Hooi, P.-S., & Chan, Y.-F. (2013). Displacement of predominant respiratory syncytial virus genotypes in Malaysia between 1989 and 2011. *Infection, Genetics and Evolution*, *14*, 357–360. https://doi.org/10.1016/j.meegid.2012.12.017

Killikelly, A. M., Kanekiyo, M., & Graham, B. S. (2016). Pre-fusion F is absent on the surface of formalin-inactivated respiratory syncytial virus. *Scientific Reports*, *6*(1), 34108. https://doi.org/10.1038/srep34108

Kim, H. J., Kim, H. S., Lee, J. M., Yoon, S. S., & Yong, D. (2016). Rapid Detection of Pseudomonas aeruginosa and Acinetobacter baumannii Harboring bla VIM-2 , bla IMP-1 and bla OXA-23 Genes by Using Loop-Mediated Isothermal Amplification Methods. *Annals of Laboratory Medicine*, *36*(1), 15. https://doi.org/10.3343/alm.2016.36.1.15

Kitagawa, Y., Orihara, Y., Kawamura, R., Imai, K., Sakai, J., Tarumoto, N., Matsuoka, M., Takeuchi, S., Maesaki, S., & Maeda, T. (2020). Evaluation of rapid diagnosis of novel coronavirus disease (COVID-19) using loop-mediated isothermal amplification. *Journal of Clinical Virology*, *129*. https://doi.org/10.1016/j.jcv.2020.104446

Klepser, D. G., Corn, C. E., Schmidt, M., Dering-Anderson, A. M., & Klepser, M. E. (2015). Health Care Resource Utilization and Costs for Influenza-like Illness Among Midwestern Health Plan Members. *Journal of Managed Care & Specialty Pharmacy*, *21*(7), 568–573. https://doi.org/10.18553/jmcp.2015.21.7.568

Kochs, G., García-Sastre, A., & Martínez-Sobrido, L. (2007). Multiple Anti-Interferon Actions of the Influenza A Virus NS1 Protein. *Journal of Virology*, *81*(13), 7011–7021. https://doi.org/10.1128/jvi.02581-06

Kolokoltsov, A. A., Deniger, D., Fleming, E. H., Roberts, N. J., Karpilow, J. M., & Davey, R. A. (2007). Small Interfering RNA Profiling Reveals Key Role of Clathrin-Mediated Endocytosis and Early Endosome Formation for Infection by Respiratory Syncytial Virus. *Journal of Virology*, *81*(14), 7786–7800. https://doi.org/10.1128/JVI.02780-06

Krammer, F., Smith, G. J. D., Fouchier, R. A. M., Peiris, M., Kedzierska, K., Doherty, P. C., Palese, P., Shaw, M. L., Treanor, J., Webster, R. G., & García-Sastre, A. (2018). Influenza. *Nature Reviews Disease Primers*, *4*(1), 1–21. https://doi.org/10.1038/s41572-018-0002-y

Krzyzaniak, M. A., Zumstein, M. T., Gerez, J. A., Picotti, P., & Helenius, A. (2013). Host Cell Entry of Respiratory Syncytial Virus Involves Macropinocytosis Followed by Proteolytic Activation of the F Protein. *PLoS Pathogens*, *9*(4), e1003309. https://doi.org/10.1371/journal.ppat.1003309

Kubo, T., Agoh, M., Mai, L. Q., Fukushima, K., Nishimura, H., Yamaguchi, A., Hirano, M., Yoshikawa, A., Hasebe, F., Kohno, S., & Morita, K. (2010). Development of a Reverse Transcription-Loop-Mediated Isothermal Amplification Assay for Detection of Pandemic (H1N1) 2009 Virus as a Novel Molecular Method for Diagnosis of Pandemic Influenza in Resource-Limited Settings. *Journal of Clinical Microbiology*, *48*(3), 728–735. https://doi.org/10.1128/JCM.01481-09

Kumar, P., Pandya, D., Singh, N., Behera, D., Aggarwal, P., & Singh, S. (2014).

Loop-mediated isothermal amplification assay for rapid and sensitive diagnosis of tuberculosis. *Journal of Infection*, *69*(6), 607–615. https://doi.org/10.1016/j.jinf.2014.08.017

Kumar, S., & Henrickson, K. J. (2012). Update on influenza diagnostics: Lessons from the novel H1N1 influenza A pandemic. *Clinical Microbiology Reviews*, *25*(2), 344–361. https://doi.org/10.1128/CMR.05016-11

Kuroda, M., Katano, H., Nakajima, N., Tobiume, M., Ainai, A., Sekizuka, T., Hasegawa, H., Tashiro, M., Sasaki, Y., Arakawa, Y., Hata, S., Watanabe, M., & Sata, T. (2010). Characterization of Quasispecies of Pandemic 2009 Influenza A Virus (A/H1N1/2009) by De Novo Sequencing Using a Next-Generation DNA Sequencer. *PLoS ONE*, *5*(4), e10256. https://doi.org/10.1371/journal.pone.0010256

Kurosaki, Y., Magassouba, N., Oloniniyi, O. K., Cherif, M. S., Sakabe, S., Takada, A., Hirayama, K., & Yasuda, J. (2016). Development and Evaluation of Reverse Transcription-Loop-Mediated Isothermal Amplification (RT-LAMP) Assay Coupled with a Portable Device for Rapid Diagnosis of Ebola Virus Disease in Guinea. *PLOS Neglected Tropical Diseases*, *10*(2), e0004472. https://doi.org/10.1371/journal.pntd.0004472

Kustin, T., Ling, G., Sharabi, S., Ram, D., Friedman, N., Zuckerman, N., Bucris, E. D., Glatman-Freedman, A., Stern, A., & Mandelboim, M. (2019). A method to identify respiratory virus infections in clinical samples using next-generation sequencing. *Scientific Reports*, *9*(1), 2606. https://doi.org/10.1038/s41598-018-37483-w

Kwanten, L., De Clerck, B., & Roymans, D. (2013). *A Fluorescence-Based High-Throughput Antiviral Compound Screening Assay Against Respiratory*

Syncytial Virus (pp. 337–344). https://doi.org/10.1007/978-1-62703-484-5_26

Lamb, L. E., Bartolone, S. N., & Chancellor, M. B. (2020). Detection of Zika Virus Using Reverse Transcription-Loop-Mediated Isothermal Amplification (RT-LAMP). *Methods in Molecular Biology*, *2142*, 137–146. https://doi.org/10.1007/978-1-0716-0581-3_12

Lamb, L. E., Bartolone, S. N., Ward, E., & Chancellor, M. B. (2020). Rapid detection of novel coronavirus/Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) by reverse transcription-loop-mediated isothermal amplification. *PLoS ONE*, *15*(6). https://doi.org/10.1371/journal.pone.0234682

Lamb, R. A., Choppin, P. W., Chanock, R. M., & Lai, C. J. (1980). Mapping of the two overlapping genes for polypeptides NS1 and NS2 on RNA segment 8 of influenza virus genome. *Proceedings of the National Academy of Sciences of the United States of America*, *77*(4 I), 1857–1861. https://doi.org/10.1073/pnas.77.4.1857

Lamb, R. A., Lai, C. J., & Choppin, P. W. (1981). Sequences of mRNAs derived from genome RNA segment 7 of influenza virus: Colinear and interrupted mRNAs code for overlapping proteins. *Proceedings of the National Academy of Sciences of the United States of America*, *78*(7 I), 4170–4174. https://doi.org/10.1073/pnas.78.7.4170

Landry, M. L., & Ferguson, D. (2014). Comparison of Simplexa Flu A/B & RSV PCR with Cytospin-Immunofluorescence and Laboratory-Developed TaqMan PCR in Predominantly Adult Hospitalized Patients. *Journal of Clinical Microbiology*, *52*(8), 3057–3059.

https://doi.org/10.1128/JCM.00738-14

Landry, Marie Louise. (2011). Diagnostic tests for influenza infection. *Current Opinion in Pediatrics*, *23*(1), 91–97. https://doi.org/10.1097/MOP.0b013e328341ebd9

Lau, Y.-L., Lai, M.-Y., Teoh, B.-T., Abd-Jamil, J., Johari, J., Sam, S.-S., Tan, K.-K., & AbuBakar, S. (2015). Colorimetric Detection of Dengue by Single Tube Reverse-Transcription-Loop-Mediated Isothermal Amplification. *PLOS ONE*, *10*(9), e0138694. https://doi.org/10.1371/journal.pone.0138694

Le Thi, N., Ikuyo, T., Nguyen Gia, B., Truong Thai, P., Vu Thi, T. Van, Bui Minh, V., Dao Xuan, C., Le Trung, D., Phan Thu, P., Do Duy, C., Pham The, T., Do, V. T., Pham Thi, P. T., Ngo Quy, C., Dang Quoc, T., Jin, T., Shohei, S., Takato, O., Noriko, N., & Tsutomu, K. (2020). A clinic-based direct real-time fluorescent reverse transcription loop-mediated isothermal amplification assay for influenza virus. *Journal of Virological Methods*, *277*, 113801. https://doi.org/10.1016/j.jviromet.2019.113801

Lee, D., Kim, E. J., Kilgore, P. E., Takahashi, H., Ohnishi, M., Tomono, J., Miyamoto, S., Omagari, D., Kim, D. W., & Seki, M. (2016). A Novel Loop-Mediated Isothermal Amplification Assay for Serogroup Identification of Neisseria meningitidis in Cerebrospinal Fluid. *Frontiers in Microbiology*, *6*. https://doi.org/10.3389/fmicb.2015.01548

Lee, H. K., Tang, J. W.-T., Loh, T. P., Kong, D. H.-L., Lau, Y.-W., Yap, H. K., & Koay, E. S.-C. (2014). Emergence of G186D Mutation in the Presence of R292K Mutation in an Immunocompromised Child Infected with Influenza A/H3N2 Virus, Treated with Oseltamivir. *Journal of Clinical Microbiology*, *52*(5), 1749–1750. https://doi.org/10.1128/JCM.00538-14

Lee, S. H., Baek, Y. H., Kim, Y.-H., Choi, Y.-K., Song, M.-S., & Ahn, J.-Y. (2016). One-Pot Reverse Transcriptional Loop-Mediated Isothermal Amplification (RT-LAMP) for Detecting MERS-CoV. *Frontiers in Microbiology*, *7*, 2166. https://doi.org/10.3389/fmicb.2016.02166

Levine, M., Sheu, T. G., Gubareva, L. V., & Mishin, V. P. (2011). Detection of Hemagglutinin Variants of the Pandemic Influenza A (H1N1) 2009 Virus by Pyrosequencing. *Journal of Clinical Microbiology*, *49*(4), 1307–1312. https://doi.org/10.1128/JCM.02424-10

Levine, S. (1977). Polypeptides of respiratory syncytial virus. *Journal of Virology*, *21*(1), 427–431. https://doi.org/10.1128/JVI.21.1.427-431.1977

Levine, S., Klaiber-Franco, R., & Paradiso, P. R. (1987). Demonstration that Glycoprotein G Is the Attachment Protein of Respiratory Syncytial Virus. *Journal of General Virology*, *68*(9), 2521–2524. https://doi.org/10.1099/0022-1317-68-9-2521

Levitz, R., Wattier, R., Phillips, P., Solomon, A., Lawler, J., Lazar, I., Weibel, C., & Kahn, J. S. (2012). Induction of IL-6 and CCL5 (RANTES) in human respiratory epithelial (A549) cells by clinical isolates of respiratory syncytial virus is strain specific. *Virology Journal*, *9*(1), 190. https://doi.org/10.1186/1743-422X-9-190

Lewis, N. S., Daly, J. M., Russell, C. A., Horton, D. L., Skepner, E., Bryant, N. A., Burke, D. F., Rash, A. S., Wood, J. L. N., Chambers, T. M., Fouchier, R. A. M., Mumford, J. A., Elton, D. M., & Smith, D. J. (2011). Antigenic and Genetic Evolution of Equine Influenza A (H3N8) Virus from 1968 to 2007. *Journal of Virology*, *85*(23), 12742–12749.

https://doi.org/10.1128/jvi.05319-11

Li, Y., Wang, S., Li, H., Song, X., Zhang, H., Duan, Y., Luo, C., Wang, B., Ji, S., Xie, Q., & Zhang, Z. (2020). Development of a convenient detection method for Trichomonas vaginalis based on loop-mediated isothermal amplification targeting adhesion protein 65. *BMC Infectious Diseases*, *20*(1), 319. https://doi.org/10.1186/s12879-020-05048-w

Liao, W., Long, D., Huang, Q., Wei, D., Liu, X., Wan, L., Feng, Y., Zhang, W., & Liu, Y. (2020). Rapid Detection to Differentiate Hypervirulent Klebsiella pneumoniae (hvKp) From Classical K. pneumoniae by Identifying peg-344 With Loop-Mediated Isothermal Amplication (LAMP). *Frontiers in Microbiology*, *11*. https://doi.org/10.3389/fmicb.2020.01189

Lifland, A. W., Jung, J., Alonas, E., Zurla, C., Crowe, J. E., & Santangelo, P. J. (2012). Human Respiratory Syncytial Virus Nucleoprotein and Inclusion Bodies Antagonize the Innate Immune Response Mediated by MDA5 and MAVS. *Journal of Virology*, *86*(15), 8245–8258. https://doi.org/10.1128/JVI.00215-12

Liljeroos, L., Krzyzaniak, M. A., Helenius, A., & Butcher, S. J. (2013). Architecture of respiratory syncytial virus revealed by electron cryotomography. *Proceedings of the National Academy of Sciences*, *110*(27), 11133–11138. https://doi.org/10.1073/pnas.1309070110

Lin, Zhen, Farooqui, A., Li, G., Wong, G. K., Mason, A. L., Banner, D., Kelvin, A. A., Kelvin, D. J., & Leon, A. J. (2014). Next-generation sequencing and bioinformatic approaches to detect and analyze influenza virus in ferrets. *The Journal of Infection in Developing Countries*, *8*(04), 498–509. https://doi.org/10.3855/jidc.3749

Lin, Zhibing, Zhang, Y., Zhang, H., Zhou, Y., Cao, J., & Zhou, J. (2012). Comparison of loop-mediated isothermal amplification (LAMP) and real-time PCR method targeting a 529-bp repeat element for diagnosis of toxoplasmosis. *Veterinary Parasitology*, *185*(2–4), 296–300. https://doi.org/10.1016/j.vetpar.2011.10.016

Lindquist, M. E., Mainou, B. A., Dermody, T. S., & Crowe, J. E. (2011). Activation of protein kinase R is required for induction of stress granules by respiratory syncytial virus but dispensable for viral replication. *Virology*, *413*(1), 103–110. https://doi.org/10.1016/j.virol.2011.02.009

Liu, H., Li, X. T., Hu, B., Zhang, L., Xue, X., Lv, S., Lu, R., Shi, N., & Yan, X. (2016). Development of Reverse Transcription Loop-Mediated Isothermal Amplification for Rapid Detection of Batai Virus in Cattle and Mosquitoes. *Vector-Borne and Zoonotic Diseases*, *16*(6), 415–422. https://doi.org/10.1089/vbz.2015.1882

Louie, J. K., Guevara, H., Boston, E., Dahlke, M., Nevarez, M., Kong, T., Schechter, R., Glaser, C. A., & Schnurr, D. P. (2010). Rapid Influenza Antigen Test for Diagnosis of Pandemic (H1N1) 2009. *Emerging Infectious Diseases*, *16*(5), 824–826. https://doi.org/10.3201/eid1605.091797

Lu, R., Wu, X., Wan, Z., Li, Y., Zuo, L., Qin, J., Jin, X., & Zhang, C. (2020). Development of a Novel Reverse Transcription Loop-Mediated Isothermal Amplification Method for Rapid Detection of SARS-CoV-2. In *Virologica Sinica* (Vol. 35, Issue 3, pp. 344–347). Science Press. https://doi.org/10.1007/s12250-020-00218-1

Lundin, A., Bergström, T., & Trybala, E. (2013). *Screening and Evaluation of*

Anti-respiratory Syncytial Virus Compounds in Cultured Cells (pp. 345–363). https://doi.org/10.1007/978-1-62703-484-5_27

Mabey, D., Peeling, R. W., Ustianowski, A., & Perkins, M. D. (2004). Diagnostics for the developing world. *Nature Reviews. Microbiology*, *2*(3), 231–240. https://doi.org/10.1038/nrmicro841

Martinello, R. A., Chen, M. D., Weibel, C., & Kahn, J. S. (2002). Correlation between Respiratory Syncytial Virus Genotype and Severity of Illness. *The Journal of Infectious Diseases*, *186*(6), 839–842. https://doi.org/10.1086/342414

Matthey, S., Nicholson, D., Ruhs, S., Alden, B., Knock, M., Schultz, K., & Schmuecker, A. (1992). Rapid detection of respiratory viruses by shell vial culture and direct staining by using pooled and individual monoclonal antibodies. *Journal of Clinical Microbiology*, *30*(3), 540–544. https://doi.org/10.1128/JCM.30.3.540-544.1992

McCurdy, L. H., & Graham, B. S. (2003). Role of Plasma Membrane Lipid Microdomains in Respiratory Syncytial Virus Filament Formation. *Journal of Virology*, *77*(3), 1747–1756. https://doi.org/10.1128/JVI.77.3.1747-1756.2003

McDonald, T. P., Pitt, A. R., Brown, G., Rixon, H. W. M., & Sugrue, R. J. (2004). Evidence that the respiratory syncytial virus polymerase complex associates with lipid rafts in virus-infected cells: a proteomic analysis. *Virology*, *330*(1), 147–157. https://doi.org/10.1016/j.virol.2004.09.034

McGivern, D. R., Collins, P. L., & Fearns, R. (2005). Identification of Internal Sequences in the 3′ Leader Region of Human Respiratory Syncytial Virus That Enhance Transcription and Confer Replication Processivity. *Journal*

of Virology, *79*(4), 2449–2460. https://doi.org/10.1128/JVI.79.4.2449-2460.2005

McLellan, J. S., Ray, W. C., & Peeples, M. E. (2013). *Structure and Function of Respiratory Syncytial Virus Surface Glycoproteins* (pp. 83–104). https://doi.org/10.1007/978-3-642-38919-1_4

Merckx, J., Wali, R., Schiller, I., Caya, C., Gore, G. C., Chartrand, C., Dendukuri, N., Papenburg, J., Rehab, W., Schiller, I., Caya, C., Gore, G. C., Chartrand, C., Dendukuri, N., & Papenburg, J. (2017). Diagnostic Accuracy of Novel and Traditional Rapid Tests for Influenza Infection Compared With Reverse Transcriptase Polymerase Chain Reaction. *Annals of Internal Medicine*, *167*(6), 394. https://doi.org/10.7326/M17-0848

Milstone, A. P. (2006). A single-season prospective study of respiratory viral infections in lung transplant recipients. *European Respiratory Journal*, *28*(1), 131–137. https://doi.org/10.1183/09031936.06.00105505

Minnucci, G., Amicarelli, G., Salmoiraghi, S., Spinelli, O., Guinea Montalvo, M. L., Giussani, U., Adlerstein, D., & Rambaldi, A. (2012). A novel, highly sensitive and rapid allele-specific loop-mediated amplification assay for the detection of the JAK2V617F mutation in chronic myeloproliferative neoplasms. *Haematologica*, *97*(9), 1394–1400. https://doi.org/10.3324/haematol.2011.056184

Mohon, A. N., Getie, S., Jahan, N., Alam, M. S., & Pillai, D. R. (2019). Ultrasensitive loop mediated isothermal amplification (US-LAMP) to detect malaria for elimination. *Malaria Journal*, *18*(1), 350. https://doi.org/10.1186/s12936-019-2979-4

Moore, C., Hibbitts, S., Owen, N., Corden, S. A., Harrison, G., Fox, J., Gelder,

C., & Westmoreland, D. (2004). Development and evaluation of a real-time nucleic acid sequence based amplification assay for rapid detection of influenza A. *Journal of Medical Virology*, *74*(4), 619–628. https://doi.org/10.1002/jmv.20221

Moreira, L. P., Watanabe, A. S. A., Carraro, E., Silva, E. R. M. da, Guatura, S. B., Granato, C., & Bellei, N. J. (2013). A survey strategy for human respiratory syncytial virus detection among haematopoietic stem cell transplant patients: epidemiological and methodological analysis. *Memórias Do Instituto Oswaldo Cruz*, *108*(1), 119–122. https://doi.org/10.1590/S0074-02762013000100021

Mori, Y., Kanda, H., & Notomi, T. (2013). Loop-mediated isothermal amplification (LAMP): Recent progress in research and development. *Journal of Infection and Chemotherapy*, *19*(3), 404–411. https://doi.org/10.1007/s10156-013-0590-0

Mori, Y., Kitao, M., Tomita, N., & Notomi, T. (2004). Real-time turbidimetry of LAMP reaction for quantifying template DNA. *Journal of Biochemical and Biophysical Methods*, *59*(2), 145–157. https://doi.org/10.1016/j.jbbm.2003.12.005

Mori, Y., Nagamine, K., Tomita, N., & Notomi, T. (2001). Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. *Biochemical and Biophysical Research Communications*, *289*(1), 150–154. https://doi.org/10.1006/bbrc.2001.5921

Nagamine, K., Hase, T., & Notomi, T. (2002). Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Molecular and*

Cellular Probes, *16*(3), 223–229. https://doi.org/10.1006/mcpr.2002.0415

Nagamine, K., Watanabe, K., Ohtsuka, K., Hase, T., & Notomi, T. (2001). Loop-mediated isothermal amplification reaction using a nondenatured template. *Clinical Chemistry*, *47*(9), 1742–1743. http://www.ncbi.nlm.nih.gov/pubmed/11514425

Nakano, R., Nakano, A., Ishii, Y., Ubagai, T., Kikuchi-Ueda, T., Kikuchi, H., Tansho-Nagakawa, S., Kamoshida, G., Mu, X., & Ono, Y. (2015). Rapid detection of the Klebsiella pneumoniae carbapenemase (KPC) gene by loop-mediated isothermal amplification (LAMP). *Journal of Infection and Chemotherapy*, *21*(3), 202–206. https://doi.org/10.1016/j.jiac.2014.11.010

Nakauchi, M., Takayama, I., Takahashi, H., Tashiro, M., & Kageyama, T. (2014). Development of a reverse transcription loop-mediated isothermal amplification assay for the rapid diagnosis of avian influenza A (H7N9) virus infection. *Journal of Virological Methods*, *204*, 101–104. https://doi.org/10.1016/j.jviromet.2014.03.028

Nakayama, T., Yamazaki, T., Yo, A., Tone, K., Mahdi Alshahni, M., Fujisaki, R., & Makimura, K. (2017). Detection of Fungi from an Indoor Environment using Loop-mediated Isothermal Amplification (LAMP) Method. *Biocontrol Science*, *22*(2), 97–104. https://doi.org/10.4265/bio.22.97

Nam, H. H., & Ison, M. G. (2019). Respiratory syncytial virus infection in adults. *BMJ*, l5021. https://doi.org/10.1136/bmj.l5021

Niihara, H., Kohno, K., Tobita, R., Ishikawa, N., & Morita, E. (2017). Rapid diagnosis of herpes zoster by loop-mediated isothermal amplification in

a pregnant woman showing folliculitis-like eruption without vesicles. *The Journal of Dermatology*, *44*(7), e174–e175. https://doi.org/10.1111/1346-8138.13861

Nkouawa, A., Sako, Y., Nakao, M., Nakaya, K., & Ito, A. (2009). Loop-Mediated Isothermal Amplification Method for Differentiation and Rapid Detection of Taenia Species. *Journal of Clinical Microbiology*, *47*(1), 168–174. https://doi.org/10.1128/JCM.01573-08

Notomi, T., Mori, Y., Tomita, N., & Kanda, H. (2015). Loop-mediated isothermal amplification (LAMP): principle, features, and future prospects. *Journal of Microbiology*, *53*(1), 1–5. https://doi.org/10.1007/s12275-015-4656-9

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., Habe, T., & Hase, T. (2000). Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research*, *28*(12), E63. https://doi.org/10.1093/nar/28.12.e63

Noton, S. L., & Fearns, R. (2015). Initiation and regulation of paramyxovirus transcription and replication. *Virology*, *479–480*, 545–554. https://doi.org/10.1016/j.virol.2015.01.014

Ocwieja, K. E., Sherrill-Mix, S., Liu, C., Song, J., Bau, H., & Bushman, F. D. (2015). A Reverse Transcription Loop-Mediated Isothermal Amplification Assay Optimized to Detect Multiple HIV Subtypes. *PLOS ONE*, *10*(2), e0117852. https://doi.org/10.1371/journal.pone.0117852

Olsen, B., Munster, V. J., Wallensten, A., Waldenström, J., Osterhaus, A. D. M. E., & Fouchier, R. A. M. (2006). Global patterns of influenza A virus in wild birds. *Science*, *312*(5772), 384–388.

https://doi.org/10.1126/science.1122438

Palese, P, & Shaw, M. (2007). Orthomyxoviridae: the viruses and their replication. In D. Knipe & P. Howley (Eds.), *Fields virology* (Philadelph).

Palese, Peter, Tumpey, T. M., & Garcia-Sastre, A. (2006). What can we learn from reconstructing the extinct 1918 pandemic influenza virus? *Immunity*, *24*(2), 121–124. https://doi.org/10.1016/j.immuni.2006.01.007

Parida, M., Posadas, G., Inoue, S., Hasebe, F., & Morita, K. (2004). Real-Time Reverse Transcription Loop-Mediated Isothermal Amplification for Rapid Detection of West Nile Virus. *Journal of Clinical Microbiology*, *42*(1), 257–263. https://doi.org/10.1128/JCM.42.1.257-263.2004

Parida, M., Sannarangaiah, S., Dash, P. K., Rao, P. V. L., & Morita, K. (2008). Loop mediated isothermal amplification (LAMP): a new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. *Reviews in Medical Virology*, *18*(6), 407–421. https://doi.org/10.1002/rmv.593

Parida, M., Shukla, J., Sharma, S., Ranghia Santhosh, S., Ravi, V., Mani, R., Thomas, M., Khare, S., Rai, A., Kant Ratho, R., Pujari, S., Mishra, B., Lakshmana Rao, P. V., & Vijayaraghavan, R. (2011). Development and Evaluation of Reverse Transcription Loop-Mediated Isothermal Amplification Assay for Rapid and Real-Time Detection of the Swine-Origin Influenza A H1N1 Virus. *The Journal of Molecular Diagnostics*, *13*(1), 100–107. https://doi.org/10.1016/j.jmoldx.2010.11.003

Paules, C., & Subbarao, K. (2017). Influenza. *The Lancet*, *390*(10095), 697–708. https://doi.org/10.1016/S0140-6736(17)30129-0

Pedneault, L., Robillard, L., & Turgeon, J. P. (1994). Validation of respiratory syncytial virus enzyme immunoassay and shell vial assay results. *Journal of Clinical Microbiology*, *32*(11), 2861–2864. https://doi.org/10.1128/JCM.32.11.2861-2864.1994

Peng, D., Xie, J., Qiang, W., Ling, K.-S., Guo, L., Fan, Z., & Zhou, T. (2017). One-step reverse transcription loop-mediated isothermal amplification assay for detection of Apple chlorotic leaf spot virus. *Journal of Virological Methods*, *248*, 154–158. https://doi.org/10.1016/j.jviromet.2017.07.002

Peret, T. C. T., Hall, C. B., Hammond, G. W., Piedra, P. A., Storch, G. A., Sullender, W. M., Tsou, C., & Anderson, L. J. (2000). Circulation Patterns of Group A and B Human Respiratory Syncytial Virus Genotypes in 5 Communities in North America. *The Journal of Infectious Diseases*, *181*(6), 1891–1896. https://doi.org/10.1086/315508

Petric, M., Comanor, L., & Petti, C. A. (2006). Role of the Laboratory in Diagnosis of Influenza during Seasonal Epidemics and Potential Pandemics. *The Journal of Infectious Diseases*, *194*(s2), S98–S110. https://doi.org/10.1086/507554

Petrova, V. N., & Russell, C. A. (2018). The evolution of seasonal influenza viruses. *Nature Reviews Microbiology*, *16*(1), 47–60. https://doi.org/10.1038/nrmicro.2017.118

Pezzi, L., Charrel, R. N., Ninove, L., Nougairede, A., Molle, G., Coutard, B., Durand, G., Leparc-Goffart, I., de Lamballerie, X., & Thirion, L. (2020). Development and Evaluation of a duo SARS-CoV-2 RT-qPCR Assay Combining Two Assays Approved by the World Health Organization

Targeting the Envelope and the RNA-Dependant RNA Polymerase (RdRp) Coding Regions. *Viruses*, *12*(6), 686. https://doi.org/10.3390/v12060686

Pham, N. T. K., Trinh, Q. D., Khamrin, P., Ukarapol, N., Kongsricharoern, T., Yamazaki, W., Komine-Aizawa, S., Okitsu, S., Maneekarn, N., Hayakawa, S., & Ushijima, H. (2015). Loop-Mediated Isothermal Amplification (LAMP) for Detection of Campylobacter jejuni and C. coli in Thai Children with Diarrhea. *Japanese Journal of Infectious Diseases*, *68*(5), 432–433. https://doi.org/10.7883/yoken.JJID.2014.450

Piedimonte, G., & Perez, M. K. (2014). Respiratory Syncytial Virus Infection and Bronchiolitis. *Pediatrics in Review*, *35*(12), 519–530. https://doi.org/10.1542/pir.35-12-519

Poon, L. L. M., Leung, C. S. W., Chan, K. H., Lee, J. H. C., Yuen, K. Y., Guan, Y., & Peiris, J. S. M. (2005). Detection of Human Influenza A Viruses by Loop-Mediated Isothermal Amplification. *Journal of Clinical Microbiology*, *43*(1), 427–430. https://doi.org/10.1128/JCM.43.1.427-430.2005

Prendergast, C., & Papenburg, J. (2013). Rapid antigen-based testing for respiratory syncytial virus: moving diagnostics from bench to bedside? *Future Microbiology*, *8*(4), 435–444. https://doi.org/10.2217/fmb.13.9

Pretorius, M. A., van Niekerk, S., Tempia, S., Moyes, J., Cohen, C., Madhi, S. A., & Venter, M. (2013). Replacement and Positive Evolution of Subtype A and B Respiratory Syncytial Virus G-Protein Genotypes From 1997–2012 in South Africa. *The Journal of Infectious Diseases*, *208*(suppl_3), S227–S237. https://doi.org/10.1093/infdis/jit477

Quesada-González, D., & Merkoçi, A. (2018). Nanomaterial-based devices for point-of-care diagnostic applications. *Chemical Society Reviews*, *47*(13),

4697–4709. https://doi.org/10.1039/C7CS00837F

Quoc, N. B., Phuong, N. D. N., Chau, N. N. B., & Linh, D. T. P. (2018). Closed tube loop-mediated isothermal amplification assay for rapid detection of hepatitis B virus in human blood. *Heliyon*, *4*(3), e00561. https://doi.org/10.1016/j.heliyon.2018.e00561

Rabalais, G. P., Stout, G. G., Ladd, K. L., & Cost, K. M. (1992). Rapid diagnosis of respiratory viral infections by using a shell vial assay and monoclonal antibody pool. *Journal of Clinical Microbiology*, *30*(6), 1505–1508. https://doi.org/10.1128/JCM.30.6.1505-1508.1992

Rabon-Stith, K. M., McGuiness, C. B., Saunders, B., Edelman, L., Kumar, V. R., & Boron, M. L. (2013). Laboratory testing trends for respiratory syncytial virus, 2007–2011. *Journal of Clinical Virology*, *58*(3), 575–578. https://doi.org/10.1016/j.jcv.2013.09.012

Rambaut, A., Pybus, O. G., Nelson, M. I., Viboud, C., Taubenberger, J. K., & Holmes, E. C. (2008). The genomic and epidemiological dynamics of human influenza A virus. *Nature*, *453*(7195), 615–619. https://doi.org/10.1038/nature06945

Rath, B., Tief, F., Obermeier, P., Tuerk, E., Karsch, K., Muehlhans, S., Adamou, E., Duwe, S., & Schweiger, B. (2012). Early detection of influenza A and B infection in infants and children using conventional and fluorescence-based rapid testing. *Journal of Clinical Virology*, *55*(4), 329–333. https://doi.org/10.1016/j.jcv.2012.08.002

Rebnord, I. K., Sandvik, H., Batman Mjelle, A., & Hunskaar, S. (2016). Out-of-hours antibiotic prescription after screening with C reactive protein: a randomised controlled study. *BMJ Open*, *6*(5), e011231.

https://doi.org/10.1136/bmjopen-2016-011231

Ren, X., Yang, F., Hu, Y., Zhang, T., Liu, L., Dong, J., Sun, L., Zhu, Y., Xiao, Y., Li, L., Yang, J., Wang, J., & Jin, Q. (2013). Full Genome of Influenza A (H7N9) Virus Derived by Direct Sequencing without Culture. *Emerging Infectious Diseases*, *19*(11). https://doi.org/10.3201/eid1911.130664

Reta, D. H., Tessema, T. S., Ashenef, A. S., Desta, A. F., Labisso, W. L., Gizaw, S. T., Abay, S. M., Melka, D. S., & Reta, F. A. (2020). Molecular and Immunological Diagnostic Techniques of Medical Viruses. *International Journal of Microbiology*, *2020*, 1–19. https://doi.org/10.1155/2020/8832728

Richard, M., Schrauwen, E. J. A., De Graaf, M., Bestebroer, T. M., Spronken, M. I. J., Van Boheemen, S., De Meulder, D., Lexmond, P., Linster, M., Herfst, S., Smith, D. J., Van Den Brand, J. M., Burke, D. F., Kuiken, T., Rimmelzwaan, G. F., Osterhaus, A. D. M. E., & Fouchier, R. A. M. (2013). Limited airborne transmission of H7N9 influenza A virus between ferrets. *Nature*, *501*(7468), 560–563. https://doi.org/10.1038/nature12476

Rima, B., Collins, P., Easton, A., Fouchier, R., Kurath, G., Lamb, R. A., Lee, B., Maisner, A., Rota, P., & Wang, L. (2017). ICTV Virus Taxonomy Profile: Pneumoviridae. *Journal of General Virology*, *98*(12), 2912–2913. https://doi.org/10.1099/jgv.0.000959

Rincheval, V., Lelek, M., Gault, E., Bouillier, C., Sitterlin, D., Blouquit-Laye, S., Galloux, M., Zimmer, C., Eleouet, J.-F., & Rameix-Welti, M.-A. (2017). Functional organization of cytoplasmic inclusion bodies in cells infected by respiratory syncytial virus. *Nature Communications*, *8*(1), 563. https://doi.org/10.1038/s41467-017-00655-9

Rivera, W. L., & Ong, V. A. (2013). Development of loop-mediated isothermal amplification for rapid detection of Entamoeba histolytica. *Asian Pacific Journal of Tropical Medicine*, *6*(6), 457–461. https://doi.org/10.1016/S1995-7645(13)60074-7

Roberts, S. R., Compans, R. W., & Wertz, G. W. (1995). Respiratory syncytial virus matures at the apical surfaces of polarized epithelial cells. *Journal of Virology*, *69*(4), 2667–2673. https://doi.org/10.1128/JVI.69.4.2667-2673.1995

Rochelet, M., Solanas, S., Grossiord, C., Maréchal, P., Résa, C., Vienney, F., Barranger, C., & Joannes, M. (2012). A thin layer-based amperometric enzyme immunoassay for the rapid and sensitive diagnosis of respiratory syncytial virus infections. *Talanta*, *100*, 139–144. https://doi.org/10.1016/j.talanta.2012.07.088

Russell, C. A., Fonville, J. M., Brown, A. E. X., Burke, D. F., Smith, D. L., James, S. L., Herfst, S., van Boheemen, S., Linster, M., Schrauwen, E. J., Katzelnick, L., Mosterin, A., Kuiken, T., Maher, E., Neumann, G., Osterhaus, A. D. M. E., Kawaoka, Y., Fouchier, R. A. M., & Smith, D. J. (2012). The Potential for Respiratory Droplet-Transmissible A/H5N1 Influenza Virus to Evolve in a Mammalian Host. *Science*, *336*(6088), 1541–1547. https://doi.org/10.1126/science.1222526

Russell, C. A., Jones, T. C., Barr, I. G., Cox, N. J., Garten, R. J., Gregory, V., Gust, I. D., Hampson, A. W., Hay, A. J., Hurt, A. C., De Jong, J. C., Kelso, A., Klimov, A. I., Kageyama, T., Komadina, N., Lapedes, A. S., Lin, Y. P., Mosterin, A., Obuchi, M., … Smith, D. J. (2008). The global circulation of seasonal influenza A (H3N2) viruses. *Science*, *320*(5874), 340–346.

https://doi.org/10.1126/science.1154137

Rutvisuttinunt, W., Chinnawirotpisan, P., Simasathien, S., Shrestha, S. K., Yoon, I.-K., Klungthong, C., & Fernandez, S. (2013). Simultaneous and complete genome sequencing of influenza A and B with high coverage by Illumina MiSeq Platform. *Journal of Virological Methods*, *193*(2), 394–404. https://doi.org/10.1016/j.jviromet.2013.07.001

Safavieh, M., Kanakasabapathy, M. K., Tarlan, F., Ahmed, M. U., Zourob, M., Asghar, W., & Shafiee, H. (2016). Emerging Loop-Mediated Isothermal Amplification-Based Microchip and Microdevice Technologies for Nucleic Acid Detection. *ACS Biomaterials Science & Engineering*, *2*(3), 278–294. https://doi.org/10.1021/acsbiomaterials.5b00449

Salinas, N. R., & Little, D. P. (2012). Electric LAMP: Virtual Loop-Mediated Isothermal AMPlification. *ISRN Bioinformatics*, *2012*, 1–5. https://doi.org/10.5402/2012/696758

Santiago-Felipe, S., Tortajada-Genaro, L. A., Carrascosa, J., Puchades, R., & Maquieira, Á. (2016). Real-time loop-mediated isothermal DNA amplification in compact disc micro-reactors. *Biosensors and Bioelectronics*, *79*, 300–306. https://doi.org/10.1016/j.bios.2015.12.045

Scharmann, U., Kirchhoff, L., Schmidt, D., Buer, J., Steinmann, J., & Rath, P.-M. (2020). Evaluation of a commercial Loop-mediated Isothermal Amplification (LAMP) assay for rapid detection of Pneumocystis jirovecii. *Mycoses*. https://doi.org/10.1111/myc.13152

Schmolke, M., & García-Sastre, A. (2010). Evasion of innate and adaptive immune responses by influenza A virus. *Cellular Microbiology*, *12*(7), 873–880. https://doi.org/10.1111/j.1462-5822.2010.01475.x

Schwartz, R. H., Selvarangan, R., & Zissman, E. N. (2015). BD Veritor System Respiratory Syncytial Virus Rapid Antigen Detection Test. *Pediatric Emergency Care*, *31*(12), 830–834. https://doi.org/10.1097/PEC.0000000000000371

Seesom, W., Thongket, P., Rattanathanawan, K., Mekseepralard, C., & Sukhumsirichart, W. (2015). Detection of Pathogenic Leptospires by Loop-Mediated Isothermal Amplification Targeting LipL32 Gene. *Journal of the Medical Association of Thailand = Chotmaihet Thangphaet*, *98 Suppl 9*, S78-84. http://www.ncbi.nlm.nih.gov/pubmed/26817214

Seki, M., Yamashita, Y., Torigoe, H., Tsuda, H., Sato, S., & Maeno, M. (2005). Loop-Mediated Isothermal Amplification Method Targeting the lytA Gene for Detection of Streptococcus pneumoniae. *Journal of Clinical Microbiology*, *43*(4), 1581–1586. https://doi.org/10.1128/JCM.43.4.1581-1586.2005

Selvarangan, R., Abel, D., & Hamilton, M. (2008). Comparison of BD Directigen™ EZ RSV and Binax NOW® RSV tests for rapid detection of respiratory syncytial virus from nasopharyngeal aspirates in a pediatric population. *Diagnostic Microbiology and Infectious Disease*, *62*(2), 157–161. https://doi.org/10.1016/j.diagmicrobio.2008.05.005

Shafik, C. F., Mohareb, E. W., & Youssef, F. G. (2011). Comparison of Direct Fluorescence Assay and Real-Time RT-PCR as Diagnostics for Respiratory Syncytial Virus in Young Children. *Journal of Tropical Medicine*, *2011*, 1–3. https://doi.org/10.1155/2011/781919

Shah, S., Bourgeois, F., Mannix, R., Nelson, K., Bachur, R., & Neuman, M. I. (2016). Emergency Department Management of Febrile Respiratory

Illness in Children. *Pediatric Emergency Care*, *32*(7), 429–434. https://doi.org/10.1097/PEC.0000000000000721

Shahriari, S., Gordon, J., & Ghildyal, R. (2016). Host cytoskeleton in respiratory syncytial virus assembly and budding. *Virology Journal*, *13*(1), 161. https://doi.org/10.1186/s12985-016-0618-z

Slinger, R., Milk, R., Gaboury, I., & Diaz-Mitoma, F. (2004). Evaluation of the QuickLab RSV Test, a New Rapid Lateral-Flow Immunoassay for Detection of Respiratory Syncytial Virus Antigen. *Journal of Clinical Microbiology*, *42*(8), 3731–3733. https://doi.org/10.1128/JCM.42.8.3731-3733.2004

Smith, M. C., Creutz, C., & Huang, Y. T. (1991). Detection of respiratory syncytial virus in nasopharyngeal secretions by shell vial technique. *Journal of Clinical Microbiology*, *29*(3), 463–465. https://doi.org/10.1128/JCM.29.3.463-465.1991

Souf, S. (2016). Recent advances in diagnostic testing for viral infections. *Bioscience Horizons*, *9*, 1–11. https://doi.org/10.1093/biohorizons/hzw010

Spinelli, O., Rambaldi, A., Rigo, F., Zanghì, P., D'Agostini, E., Amicarelli, G., Colotta, F., Divona, M., Ciardi, C., Lo Coco, F., & Minnucci, G. (2015). Simple, rapid and accurate molecular diagnosis of acute promyelocytic leukemia by loop mediated amplification technology. *Oncoscience*, *2*(1), 50–58. https://doi.org/10.18632/oncoscience.114

Srinivasakumar, N., Ogra, P. L., & Flanagan, T. D. (1991). Characteristics of fusion of respiratory syncytial virus with HEp-2 cells as measured by R18 fluorescence dequenching assay. *Journal of Virology*, *65*(8), 4063–4069.

https://doi.org/10.1128/JVI.65.8.4063-4069.1991

Stöhr, K. (2002). Influenza—WHO cares. *The Lancet Infectious Diseases*, *2*(9), 517. https://doi.org/10.1016/S1473-3099(02)00366-3

Sun, J., Najafzadeh, M. J. J., Vicente, V., Xi, L., & de Hoog, G. S. S. (2010). Rapid detection of pathogenic fungi using loop-mediated isothermal amplification, exemplified by Fonsecaea agents of chromoblastomycosis. *Journal of Microbiological Methods*, *80*(1), 19–24. https://doi.org/10.1016/j.mimet.2009.10.002

Tabatabai, J., Prifert, C., Pfeil, J., Grulich-Henn, J., & Schnitzler, P. (2014). Novel Respiratory Syncytial Virus (RSV) Genotype ON1 Predominates in Germany during Winter Season 2012–13. *PLoS ONE*, *9*(10), e109191. https://doi.org/10.1371/journal.pone.0109191

Takano, C., Kuramochi, Y., Seki, M., Kim, D. W., Omagari, D., Sasano, M., Chang, B., Ohnishi, M., Kim, E. J., Fuwa, K., Kilgore, P. E., Hoshino, T., & Hayakawa, S. (2019). Molecular serotype-specific identification of Streptococcus pneumoniae using loop-mediated isothermal amplification. *Scientific Reports*, *9*(1), 19823. https://doi.org/10.1038/s41598-019-56225-0

Taleb, S. A., Al Thani, A. A., Al Ansari, K., & Yassine, H. M. (2018). Human respiratory syncytial virus: pathogenesis, immune responses, and current vaccine approaches. *European Journal of Clinical Microbiology & Infectious Diseases*, *37*(10), 1817–1827. https://doi.org/10.1007/s10096-018-3289-4

Teng, M. N., & Collins, P. L. (1998). Identification of the Respiratory Syncytial Virus Proteins Required for Formation and Passage of Helper-Dependent

Infectious Particles. *Journal of Virology*, *72*(7), 5707–5716. https://doi.org/10.1128/JVI.72.7.5707-5716.1998

Thai, H. T. C., Le, M. Q., Vuong, C. D., Parida, M., Minekawa, H., Notomi, T., & Hasebe, F. (2004). Development and Evaluation of a Novel Loop-Mediated Isothermal Amplification Method for Rapid Detection of Severe Acute Respiratory Syndrome Coronavirus Development and Evaluation of a Novel Loop-Mediated Isothermal Amplification Method for Rapid Detectio. *Journal of Clinical Microbiology*, *42*(5), 1956–1961. https://doi.org/10.1128/JCM.42.5.1956

Thevenin, T., Lobert, P. E., Dewilde, A., & Hober, D. (2012). A respiratory syncytial virus isolate enables the testing of virucidal products. *International Journal of Hygiene and Environmental Health*, *215*(3), 293–296. https://doi.org/10.1016/j.ijheh.2011.10.001

Thompson, W. W., Shay, D. K., Weintraub, E., Brammer, L., Cox, N., Anderson, L. J., & Fukuda, K. (2003). Mortality Associated With Influenza and Respiratory Syncytial Virus in the United States. *JAMA*, *289*(2), 179. https://doi.org/10.1001/jama.289.2.179

Tirloni, E., Bernardi, C., Drago, S., Stampone, G., Pomilio, F., Cattaneo, P., & Stella, S. (2017). Evaluation of a loop-mediated isothermal amplification method for the detection of Listeria monocytogenes in dairy food. *Italian Journal of Food Safety*, *6*(4), 6890. https://doi.org/10.4081/ijfs.2017.6890

Tone, K., Suzuki, J., Alshahni, M. M., Kuwano, K., & Makimura, K. (2019). Species-specific detection of medically important aspergilli by a loop-mediated isothermal amplification method in chronic pulmonary

aspergillosis. *Medical Mycology*, *57*(6), 703–709. https://doi.org/10.1093/mmy/myy128

Tong, S., Zhu, X., Li, Y., Shi, M., Zhang, J., Bourgeois, M., Yang, H., Chen, X., Recuenco, S., Gomez, J., Chen, L. M., Johnson, A., Tao, Y., Dreyfus, C., Yu, W., McBride, R., Carney, P. J., Gilbert, A. T., Chang, J., … Donis, R. O. (2013). New World Bats Harbor Diverse Influenza A Viruses. *PLoS Pathogens*, *9*(10). https://doi.org/10.1371/journal.ppat.1003657

Townsend, M. B., Dawson, E. D., Mehlmann, M., Smagala, J. A., Dankbar, D. M., Moore, C. L., Smith, C. B., Cox, N. J., Kuchta, R. D., & Rowlen, K. L. (2006). Experimental Evaluation of the FluChip Diagnostic Microarray for Influenza Virus Surveillance. *Journal of Clinical Microbiology*, *44*(8), 2863–2871. https://doi.org/10.1128/JCM.00134-06

Tripp, R. A., Alvarez, R., Anderson, B., Jones, L., Weeks, C., & Chen, W. (2007). Bioconjugated nanoparticle detection of respiratory syncytial virus infection. *International Journal of Nanomedicine*, *2*(1), 117–124. https://doi.org/10.2147/nano.2007.2.1.117

Trombetta, V. K., Chan, Y. L., & Bankowski, M. J. (2018). Are Rapid Influenza Antigen Tests Still Clinically Useful in Today's Molecular Diagnostics World? *Hawai'i Journal of Medicine & Public Health : A Journal of Asia Pacific Medicine & Public Health*, *77*(9), 226–230. http://www.ncbi.nlm.nih.gov/pubmed/30221077

Tuttle, R., Weick, A., Schwarz, W. S., Chen, X., Obermeier, P., Seeber, L., Tief, F., Muehlhans, S., Karsch, K., Peiser, C., Duwe, S., Schweiger, B., & Rath, B. (2015). Evaluation of novel second-generation RSV and influenza rapid tests at the point of care. *Diagnostic Microbiology and Infectious*

Disease, *81*(3), 171–176. https://doi.org/10.1016/j.diagmicrobio.2014.11.013

Uddin, S., Ibrahim, F., Sayad, A., Thiha, A., Pei, K., Mohktar, M., Hashim, U., Cho, J., & Thong, K. (2015). A Portable Automatic Endpoint Detection System for Amplicons of Loop Mediated Isothermal Amplification on Microfluidic Compact Disk Platform. *Sensors*, *15*(3), 5376–5389. https://doi.org/10.3390/s150305376

Uemura, N., Makimura, K., Onozaki, M., Otsuka, Y., Shibuya, Y., Yazaki, H., Kikuchi, Y., Abe, S., & Kudoh, S. (2008). Development of a loop-mediated isothermal amplification method for diagnosing Pneumocystis pneumonia. *Journal of Medical Microbiology*, *57*(1), 50–57. https://doi.org/10.1099/jmm.0.47216-0

Ushio, M., Yui, I., Yoshida, N., Fujino, M., Yonekawa, T., Ota, Y., Notomi, T., & Nakayama, T. (2005). Detection of respiratory syncytial virus genome by subgroups-A, B specific reverse transcription loop-mediated isothermal amplification (RT-LAMP). *Journal of Medical Virology*, *77*(1), 121–127. https://doi.org/10.1002/jmv.20424

Utley, T. J., Ducharme, N. A., Varthakavi, V., Shepherd, B. E., Santangelo, P. J., Lindquist, M. E., Goldenring, J. R., & Crowe, J. E. (2008). Respiratory syncytial virus uses a Vps4-independent budding mechanism controlled by Rab11-FIP2. *Proceedings of the National Academy of Sciences*, *105*(29), 10209–10214. https://doi.org/10.1073/pnas.0712144105

Valdovinos, M. R., & Gómez, B. (2003). Establishment of Respiratory Syncytial Virus Persistence in Cell Lines: Association with Defective Interfering Particles. *Intervirology*, *46*(3), 190–198.

https://doi.org/10.1159/000071461

Van der Sandt, C. E., Kreijtz, J. H. C. M., & Rimmelzwaan, G. F. (2012). Evasion of influenza A viruses from innate and adaptive immune responses. *Viruses*, *4*(9), 1438–1476. https://doi.org/10.3390/v4091438

Van Riel, D., Munster, V. J., De Wit, E., Rimmelzwaan, G. F., Fouchier, R. A. M., Osterhaus, A. D. M. E., & Kuiken, T. (2006). H5N1 virus attachment to lower respiratory tract. *Science*, *312*(5772), 399. https://doi.org/10.1126/science.1125548

Vandini, S., Biagi, C., & Lanari, M. (2017). Respiratory Syncytial Virus: The Influence of Serotype and Genotype Variability on Clinical Course of Infection. *International Journal of Molecular Sciences*, *18*(8), 1717. https://doi.org/10.3390/ijms18081717

Varsha, V., Aishwarya, S., Murchana, S., Naveen, G., Ramya, M., & Rathinasabapathi, P. (2020). Correction pen based paper fluidic device for the detection of multiple gene targets of Leptospira using Loop Mediated Isothermal Amplification. *Journal of Microbiological Methods*, *174*, 105962. https://doi.org/10.1016/j.mimet.2020.105962

Vekemans, J., Moorthy, V., Giersing, B., Friede, M., Hombach, J., Arora, N., Modjarrad, K., Smith, P. G., Karron, R., Graham, B., & Kaslow, D. C. (2019). Respiratory syncytial virus vaccine research and development: World Health Organization technological roadmap and preferred product characteristics. *Vaccine*, *37*(50), 7394–7395. https://doi.org/10.1016/j.vaccine.2017.09.092

Vemula, S., Zhao, J., Liu, J., Wang, X., Biswas, S., & Hewlett, I. (2016). Current Approaches for Diagnosis of Influenza Virus Infections in Humans.

Viruses, *8*(4), 96. https://doi.org/10.3390/v8040096

Venter, M., Madhi, S. A., Tiemessen, C. T., & Schoub, B. D. (2001). Genetic diversity and molecular epidemiology of respiratory syncytial virus over four consecutive seasons in South Africa: identification of new subgroup A and B genotypes The GenBank accession numbers of the sequences reported in this paper are AF348802–. *Journal of General Virology*, *82*(9), 2117–2124. https://doi.org/10.1099/0022-1317-82-9-2117

Walsh, E. E. (2017). Respiratory Syncytial Virus Infection. *Clinics in Chest Medicine*, *38*(1), 29–36. https://doi.org/10.1016/j.ccm.2016.11.010

Walsh, E. E., & Hall, C. B. (2015). *Respiratory Syncytial Virus (RSV)* (El Sevier (ed.); 8th ed.).

Walsh, P., Overmyer, C., Hancock, C., Heffner, J., Walker, N., Nguyen, T., Shanholtzer, L., Caldera, E., Pusavat, J., Mordechai, E., Adelson, M. E., & Iacono, K. T. (2014). Is the interpretation of rapid antigen testing for respiratory syncytial virus as simple as positive or negative? *Emergency Medicine Journal*, *31*(2), 153–159. https://doi.org/10.1136/emermed-2013-202729

Wang, J., Tai, W., Angione, S. L., John, A. R., Opal, S. M., Artenstein, A. W., & Tripathi, A. (2013). Subtyping Clinical Specimens of Influenza A Virus by Use of a Simple Method To Amplify RNA Targets. *Journal of Clinical Microbiology*, *51*(10), 3324–3330. https://doi.org/10.1128/JCM.01206-13

Wang, Q., Zhou, Y., Li, S., Zhuo, C., Xu, S., Huang, L., Yang, L., & Liao, K. (2013). Real-Time Fluorescence Loop Mediated Isothermal Amplification for the Detection of Acinetobacter baumannii. *PLoS ONE*, *8*(7), e66406.

https://doi.org/10.1371/journal.pone.0066406

Wang, X., Li, X., Hu, S., Qu, H., Zhang, Y., Ni, H., & Wang, X. (2015). Rapid detection of active human cytomegalovirus infection in pregnancy using loop-mediated isothermal amplification. *Molecular Medicine Reports*, *12*(2), 2269–2274. https://doi.org/10.3892/mmr.2015.3572

Webster, R. G., Bean, W. J., Owen, G. T., Chambers, T. M., & Kawaoka, Y. (2014). Evolution and ecology of influenza a viruses. *Current Topics in Microbiology and Immunology*, *385*(1), 359–375. https://doi.org/10.1007/82_2014_396

Weinberg, A., Lyu, D. M., Li, S., Marquesen, J., & Zamora, M. R. (2010). Incidence and morbidity of human metapneumovirus and other community-acquired respiratory viruses in lung transplant recipients. *Transplant Infectious Disease*, *12*(4), 330–335. https://doi.org/10.1111/j.1399-3062.2010.00509.x

White, L. J., Waris, M., Cane, P. A., Nokes, D. J., & Medley, G. F. (2005). The transmission dynamics of groups A and B human respiratory syncytial virus (hRSV) in England & Wales and Finland: seasonality and cross-protection. *Epidemiology and Infection*, *133*(2), 279–289. https://doi.org/10.1017/S0950268804003450

Whitehead, T. A., Chevalier, A., Song, Y., Dreyfus, C., Fleishman, S. J., De Mattos, C., Myers, C. A., Kamisetty, H., Blair, P., Wilson, I. A., & Baker, D. (2012). Optimization of affinity, specificity and function of designed influenza inhibitors using deep sequencing. *Nature Biotechnology*, *30*(6), 543–548. https://doi.org/10.1038/nbt.2214

Widmer, K., Griffin, M. R., Zhu, Y., Williams, J. V., & Talbot, H. K. (2014).

Respiratory syncytial virus- and human metapneumovirus-associated emergency department and hospital burden in adults. *Influenza and Other Respiratory Viruses*, *8*(3), 347–352. https://doi.org/10.1111/irv.12234

Widmer, K., Zhu, Y., Williams, J. V., Griffin, M. R., Edwards, K. M., & Talbot, H. K. (2012). Rates of Hospitalizations for Respiratory Syncytial Virus, Human Metapneumovirus, and Influenza Virus in Older Adults. *The Journal of Infectious Diseases*, *206*(1), 56–62. https://doi.org/10.1093/infdis/jis309

Wong, Y.-P., Othman, S., Lau, Y.-L., Radu, S., & Chee, H.-Y. (2018). Loop-mediated isothermal amplification (LAMP): a versatile technique for detection of micro-organisms. *Journal of Applied Microbiology*, *124*(3), 626–643. https://doi.org/10.1111/jam.13647

World Health Organisation (WHO). (2020). *Burden of disease*. https://www.who.int/influenza/surveillance_monitoring/bod/en/

Wright, P. F., Ikizler, M. R., Gonzales, R. A., Carroll, K. N., Johnson, J. E., & Werkhaven, J. A. (2005). Growth of Respiratory Syncytial Virus in Primary Epithelial Cells from the Human Respiratory Tract. *Journal of Virology*, *79*(13), 8651–8654. https://doi.org/10.1128/JVI.79.13.8651-8654.2005

Wu, L.-T., Curran, M. D., Ellis, J. S., Parmar, S., Ritchie, A. V., Sharma, P. I., Allain, J.-P., Jalal, H., Zambon, M., & Lee, H. H. (2010). Nucleic Acid Dipstick Test for Molecular Diagnosis of Pandemic H1N1. *Journal of Clinical Microbiology*, *48*(10), 3608–3613. https://doi.org/10.1128/JCM.00981-10

Wu, L.-T., Thomas, I., Curran, M. D., Ellis, J. S., Parmar, S., Goel, N., Sharma, P.

I., Allain, J.-P., & Lee, H. H. (2013). Duplex Molecular Assay Intended for Point-of-Care Diagnosis of Influenza A/B Virus Infection. *Journal of Clinical Microbiology*, *51*(9), 3031–3038. https://doi.org/10.1128/JCM.00740-13

Xia, Y., Guo, X.-G., & Zhou, S. (2014). Rapid detection of Streptococcus pneumoniae by real-time fluorescence loop-mediated isothermal amplification. *Journal of Thoracic Disease*, *6*(9), 1193–1199. https://doi.org/10.3978/j.issn.2072-1439.2014.07.29

Yamada, S., Suzuki, Y., Suzuki, T., Le, M. Q., Nidom, C. A., Sakai-Tagawa, Y., Muramoto, Y., Ito, M., Kiso, M., Horimoto, T., Shinya, K., Sawada, T., Kiso, M., Usui, T., Murata, T., Lin, Y., Hay, A., Haire, L. F., Stevens, D. J., … Kawaoka, Y. (2006). Haemagglutinin mutations responsible for the binding of H5N1 influenza A viruses to human-type receptors. *Nature*, *444*(7117), 378–382. https://doi.org/10.1038/nature05264

Yang, C., Li, X., Li, H., Guo, C., Li, Y., & Liu, Z. (2019). Distinguishing Four HCV Genotypes by Loop Mediated Isothermal Amplification Assay. *Clinical Laboratory*, *65*(11/2019). https://doi.org/10.7754/Clin.Lab.2019.190142

Yang, L., Chan, K. H., Suen, L. K. P., Chan, K. P., Wang, X., Cao, P., He, D., Peiris, J. S. M., & Wong, C. M. (2015). Age-specific epidemic waves of influenza and respiratory syncytial virus in a subtropical city. *Scientific Reports*, *5*, 1–8. https://doi.org/10.1038/srep10390

Yang, W., Pin, C., Haibing, G., Yang, C., Hui, L., & Qigai, H. (2009). Loop-mediated isothermal amplification targeting the apxIVA gene for detection of Actinobacillus pleuropneumoniae. *FEMS Microbiology*

Letters, *300*(1), 83–89. https://doi.org/10.1111/j.1574-6968.2009.01779.x

Ye, L., Li, Y., Zhao, J., Zhang, Z., Meng, H., Yan, H., Miyoshi, S. -i., & Shi, L. (2015). Development of a real-time loop-mediated isothermal amplification assay for the sensitive and rapid detection of Listeria monocytogenes. *Letters in Applied Microbiology*, *61*(1), 85–90. https://doi.org/10.1111/lam.12429

Yeo, D. S.-Y., Chan, R., Brown, G., Ying, L., Sutejo, R., Aitken, J., Tan, B.-H., Wenk, M. R., & Sugrue, R. J. (2009). Evidence that selective changes in the lipid composition of raft-membranes occur during respiratory syncytial virus infection. *Virology*, *386*(1), 168–182. https://doi.org/10.1016/j.virol.2008.12.017

Yoda, T., Suzuki, Y., Yamazaki, K., Sakon, N., Kanki, M., Kase, T., Takahashi, K., & Inoue, K. (2009). Application of a modified loop-mediated isothermal amplification kit for detecting norovirus genogroups I and II. *Journal of Medical Virology*, *81*(12), 2072–2078. https://doi.org/10.1002/jmv.21626

Yu, H., Alonso, W. J., Feng, L., Tan, Y., Shu, Y., Yang, W., & Viboud, C. (2013). Characterization of Regional Influenza Seasonality Patterns in China and Implications for Vaccination Strategies: Spatio-Temporal Modeling of Surveillance Data. *PLoS Medicine*, *10*(11). https://doi.org/10.1371/journal.pmed.1001552

Zebedee, S. L., & Lamb, R. A. (1988). Influenza A virus M2 protein: monoclonal antibody restriction of virus growth and detection of M2 in virions. *Journal of Virology*, *62*(8), 2762–2772.

https://doi.org/10.1128/jvi.62.8.2762-2772.1988

Zhang, H., Thekisoe, O. M. M., Aboge, G. O., Kyan, H., Yamagishi, J., Inoue, N., Nishikawa, Y., Zakimi, S., & Xuan, X. (2009). Toxoplasma gondii: Sensitive and rapid detection of infection by loop-mediated isothermal amplification (LAMP) method. *Experimental Parasitology*, *122*(1), 47–50. https://doi.org/10.1016/j.exppara.2009.01.012

Zhang, L., Peeples, M. E., Boucher, R. C., Collins, P. L., & Pickles, R. J. (2002). Respiratory Syncytial Virus Infection of Human Airway Epithelial Cells Is Polarized, Specific to Ciliated Cells, and without Obvious Cytopathology. *Journal of Virology*, *76*(11), 5654–5666. https://doi.org/10.1128/JVI.76.11.5654-5666.2002

Zhang, N., Wang, L., Deng, X., Liang, R., Su, M., He, C., Hu, L., Su, Y., Ren, J., Yu, F., Du, L., & Jiang, S. (2020). Recent advances in the detection of respiratory virus infection in humans. *Journal of Medical Virology*, *92*(4), 408–417. https://doi.org/10.1002/jmv.25674

Zhang, P., Vemula, S. V., Zhao, J., Du, B., Mohan, H., Liu, J., El Mubarak, H. S., Landry, M. L., & Hewlett, I. (2014). A Highly Sensitive Europium Nanoparticle-Based Immunoassay for Detection of Influenza A/B Virus Antigen in Clinical Specimens. *Journal of Clinical Microbiology*, *52*(12), 4385–4387. https://doi.org/10.1128/JCM.02635-14

Zhang, Y., Yao, Y., Du, W., Wu, K., Xu, W., Lin, M., Tan, H., & Li, J. (2017). Development of loop-mediated isothermal amplification with Plasmodium falciparum unique genes for molecular diagnosis of human malaria. *Pathogens and Global Health*, *111*(5), 247–255. https://doi.org/10.1080/20477724.2017.1347379

Zhao, J., Ragupathy, V., Liu, J., Wang, X., Vemula, S. V., El Mubarak, H. S., Ye, Z., Landry, M. L., & Hewlett, I. (2015). Nanomicroarray and Multiplex Next-Generation Sequencing for Simultaneous Identification and Characterization of Influenza Viruses. *Emerging Infectious Diseases*, *21*(3). https://doi.org/10.3201/eid2103.141169

Zhao, N., Liu, J., & Sun, D. (2017). Detection of HCV genotypes 1b and 2a by a reverse transcription loop-mediated isothermal amplification assay. *Journal of Medical Virology*, *89*(6), 1048–1054. https://doi.org/10.1002/jmv.24747

Zhou, J., Liao, Y., Li, H., Lu, X., Han, X., Tian, Y., Chen, S., & Yang, R. (2015). Development of a Loop-Mediated Isothermal Amplification Assay for Rapid Detection of Trichosporon asahii in Experimental and Clinical Samples. *BioMed Research International*, *2015*, 1–9. https://doi.org/10.1155/2015/732573

Zhou, Y., Wan, Z., Yang, S., Li, Y., Li, M., Wang, B., Hu, Y., Xia, X., Jin, X., Yu, N., & Zhang, C. (2019). A Mismatch-Tolerant Reverse Transcription Loop-Mediated Isothermal Amplification Method and Its Application on Simultaneous Detection of All Four Serotype of Dengue Viruses. *Frontiers in Microbiology*, *10*. https://doi.org/10.3389/fmicb.2019.01056

Ziros, P. G., Kokkinos, P. A., Allard, A., & Vantarakis, A. (2015). Development and Evaluation of a Loop-Mediated Isothermal Amplification Assay for the Detection of Adenovirus 40 and 41. *Food and Environmental Virology*, *7*(3), 276–285. https://doi.org/10.1007/s12560-015-9182-8

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- that this Agreement contains specific clauses for the legal protection of the Study Program results and specific confidentiality constraints on such results;

☐ **Case 2: The tutor and the student intend to file a patent application**
- that the doctoral thesis has elements and solutions of great technological relevance, which seem to meet the inventiveness requirements of novelty, originality and industrial applicability as validated/acknowledged by the Tutor Prof. .....;
- that he/she intends to legally protect the invention covered by the thesis, due to its outstanding and significant commercial potential, by filing a specific patent application:

FOR INTELLECTUAL PROPERTY (three cases)

☐ **Case 1: use and commercial exploitation**
- that the doctoral thesis, in addition to satisfying the intellectual property requirements of novelty and originality in the field of ....., as validated/acknowledged by the Tutor Prof ..... also has strong, tangible and immediate prospects of being used and commercially exploited;

☐ **Case 2: publishing contract**
- that the undersigned has signed/ intends to sign a specific publishing contract, by which the publishing rights of the thesis or extracts thereof have been transferred and which contains specific guarantee clauses for the transferred rights;

☒ **Case 3 : implementation of commercial potential**
- he/she intends to commercially use the intellectual work by *(specify mode)* DISSEMINATION thereby tangibly implementing its commercial potential;

OBSERVES

- that by virtue of the above, it is appropriate and necessary to postpone the publication of the thesis through the Open Archives system for a period of 36 months (max 36), as of the PhD graduation date;

ACKNOWLEDGES

- that pursuant to art. 6, paragraph 11 of Ministerial Decree of 30 April 1999, ref. no. 224/1999, the University is under the obligation to ensure that a copy of the thesis is filed at the Central National Libraries of Rome and Florence, such filing to be implemented pursuant to and in accordance with Law no. 106 of 15 April 2004 and the related Implementing Regulation (Presidential Decree no. 252 of 3 May 2006);

HEREBY REQUESTS

- that the University postpone the publication of the thesis through the Open Archives system and files it with the Central National Libraries of Rome and Florence in accordance with the aforementioned law.

Milan, 28/10/2020

For more information on the embargo, please contact :
Research Area - Commercial use of research
Sector Tel. 02/6448.6381

Student Signature
Chiara Brambati

Coordinator Signature
PAOLA BRANDUARDI

Tutor Signature
Mario Dalmi

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Form drawn up on 6th February 2019

UNIVERSITA' DEGLI STUDI DI MILANO-BICOCCA

Upload during the submission of the final application

REQUEST FOR EMBARGO OF THESIS
Research Doctorate (PhD)

Registration No. 836736

Pursuant to art. 19 paragraph 10 of the Regulations for Research Doctorate (PhD) courses of the University of Milano - Bicocca

The undersigned BRAMBATI CHIARA born in TREVIGLIO (BG) on 23/01/1990 enrolled in the Doctorate course in SCIENCES, TECHNOLOGIES FOR BIOMOLECULAR SYSTEMS (cycle XXXIII) (33°)

MAKING REFERENCE TO AND CONFIRMING

the statement delivered by the undersigned to this Office, for the self-archiving of the above mentioned thesis,

being aware that, pursuant to Presidential Decree no. 445 of 28.12.2000, false statements, forging documents and using forged documents are punishable under the Italian Criminal Code and the Italian special applicable laws

HEREBY STATES *(please tick as applicable)*

FOR INDUSTRIAL PROPERTY (two cases)

☐ **case 1: there is a contract with an external lender**
- that the doctoral thesis has elements and solutions of great technological relevance, which seem to meet the inventiveness requirements of novelty, originality and industrial applicability as validated/acknowledged by the Tutor Prof. .....;
- that the scholarship for the Doctorate Course (PhD) being pursued by the undersigned is financially supported by *(specify funding entity)* .....;
- that the University has signed a special agreement with the Funding entity of the Doctorate Scholarship;
- that this Agreement contains specific clauses for the legal protection of the Study Program results and specific confidentiality constraints on such results;

☐ **Case 2: The tutor and the student intend to file a patent application**
- that the doctoral thesis has elements and solutions of great technological relevance, which seem to meet the inventiveness requirements of novelty, originality and industrial applicability as validated/acknowledged by the Tutor Prof. .....;
- that he/she intends to legally protect the invention covered by the thesis, due to its outstanding and significant commercial potential, by filing a specific patent application:

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