# Improving the identification of influenza A virus from wild bird faecal samples in South Africa

By

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# DECLARATION

I, Thandeka Precious Phiri declare that the dissertation submitted to the University of Pretoria for an MSc degree in Veterinary Science at the Department of Production Animal Studies and the content is my original work and has not previously been submitted for a degree at any tertiary institution.

Signature: _____

Date: _____

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- Family

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# SUMMARY

Influenza A virus (IAV) is a single-stranded negative-sense RNA virus that is a member of the *Orthomyxoviridae* group. IAV has been detected in over 100 bird species from 26 different families, although *Anseriformes* and *Charadriiformes* are considered the natural host of the virus. Surveillance of wild birds for IAV is important as it plays a role in the early detection system for the introduction of potentially dangerous IAV strains with the ability to cause damage in the poultry industry, or even affect human health. Fresh wild bird faecal samples at a wild ducks' roosts may contain a high concentration of IAV. A method developed by Zhou *et al.* in 2009 described the amplification of full IAV genome in a single tube, and this method has been successfully employed at the University of Pretoria (UP) in the subtyping of IAV in clinical samples (organs and tracheal swabs). However, when applied to environmental faecal samples the technique was unsuccessful, presumably due to PCR inhibitors and a high level of contaminating nucleic acids from bacteria. Therefore, the first objective of this study was to optimize the IAV pan-genome RT-PCR for environmental faecal samples. PCR parameters such as $MgSO_4$ concentration, annealing temperatures, and different PCR reagents were optimized on spiked faecal samples. The second objective was to screen fresh environmental faecal samples from wild duck at a site in Pretoria to identify positive field samples for testing. A total of 2,144 faecal swabs were collected from January through-February 2021 and screened with IAV-specific real-time RT-PCR assay. Two samples with positive results were submitted to the Central Analytical Facility in Stellenbosch University for Ion Torrent Next-Generation Sequencing. After assembling the results in the CLC Genomics Workbench software and verifying the results by BLAST analysis, TP2118 was conclusively identified as an H9N2 strain, but whereas the presence of IAV-specific internal genes could be identified for TP2067, the sequence data was insufficient to identify the subtype. TP2118 represents the first H9N2 virus ever detected in wild ducks in Gauteng Province.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| % | Percentage |
| µl | Microliters |
| ABI | Applied Biosystem Instrument |
| AIV | Avian influenza virus |
| Bp | Base pair |
| BLAST | Basic Local Alignment Search Tool |
| Ct | Cycle threshold |
| dNTPs | Deoxynucleoside triphosphate synthesis |
| G | Grams |
| HA | Hemagglutinin |
| HPAI | High pathogenic avian influenza |
| IAV | Influenza A virus |
| Kb | Kilo base |
| KZN | KwaZulu Natal |
| LPAI | Low pathogenic avian influenza |
| $MgSO_4$ | Magnesium Sulphate |
| Min | Minutes |
| Ml | Millilitres |
| mM | Millimolar |
| M1+M2 | Matrix protein 1 and 2 |

| | |
|---|---|
| M RTPCR | multisegmented real time PCR |
| NA | Neuraminidase |
| Nm | Nanometers |
| NGS | Next generation sequencing |
| NP | Nucleoprotein |
| NS | Non-structural protein |
| Nt | Nucleotide |
| NTC | Negative template control |
| PA | Polymerase Acid |
| PB1 | Polymerase basic protein 1 |
| PB1-F2 | Polymerase basic 1 F2 |
| PB2 | Polymerase basic protein 2 |
| pmole | Picomole |
| RCF | relative centrifugal force |
| RNP | Ribonucleoprotein |
| Rpm | Revolutions per minute |
| rRT-PCR | reverse Real Time Polymerase Chain Reaction |
| RT | Room Temperature |
| Sec | Seconds |
| ssRNA | single stranded RNA |
| vRNA | Viral RNA |
| vRNP | Viral ribonucleoprotein |
| VTM | Viral Transport Medium |

# CHAPTER 1: LITERATURE REVIEW

## 1.1. Classification

The *Orthomyxoviridae* is a biological family of viruses that contain of enveloped negative sense single stranded RNA (ssRNA) segmented genomes. There are seven separate genera in the group; four of which are important influenza virus representatives namely : *Alphainfluenzavirus* (influenza A virus); *Betainfluenzavirus* (influenza B virus); *Gammainfluenzavirus* (influenza C virus) and the newly discovered *Deltainfluenzavirus* (influenza D virus). Each viral subtype is defined based on antigenic differences (Sangkakam et al., 2021, Nelson, 2022). Influenza virus type A (IAV) causes the disease Avian Influenza as it affects and infects the avian species but it has been found to infect humans and other mammals (swine and horses) causing significant morbidity and mortality (Suarez and Schultz-Cherry, 2000, McAuley et al., 2019). Influenza B and C virus are found in humans, however type B has also been found to infect seals and cause annual epidemics in people. Influenza C virus has also been detected in swine although reassortment has been detected in the virus; it does not cause serious disease and results in mild asymptomatic infections in both host species (humans and swine). The recently discovered influenza D virus was detected in cows and bats. Neither influenza B,C or D viruses have been detected in avian species thus far (Klenk et al., 2008, Sangkakam et al., 2021, Nelson, 2022).

## 1.2. Influenza A virus genome structure

Influenza A virus (IAV) is a spherical pleomorphic virus that ranges between 80 to 120 nm in diameter (Figure 1). The IAV is composed of eight different genome segments ranging from 2340 to 890 nucleotides totalling approximately 13.5 kb and encoding for at least eleven different genes (Suarez and Schultz-Cherry, 2000). Polymerase basic (PB1 and PB2), polymerase acid (PA), hemagglutinin (HA), neuraminidase (NA), matrix protein (M1), membrane ion channel (M2), nucleoprotein (NP) and nonstructural proteins (NS1 and NS2) are the encoded in the viral genome segments. There are three surface proteins in the viral structure (M2, HA and NA) and five internal proteins (NP, M1, PA, PB1 and PB2), the latter forming the components of the ribonucleoprotein (RNP). The

RNP, composed of viral RNA, an RNA segment dependent RNA polymerase complex, and multiple nucleoproteins form a helical rod-like shaped structure with a uniform diameter of approximately 12 nm ((Nakatsu et al., 2016). Nucleotides at both ends of the viral genome serve as promoters for replication and transcription. When the viral genome is reassorted it results in the spread of novel viruses among bird species (Abolnik et al., 2007, Nakatsu et al., 2016).

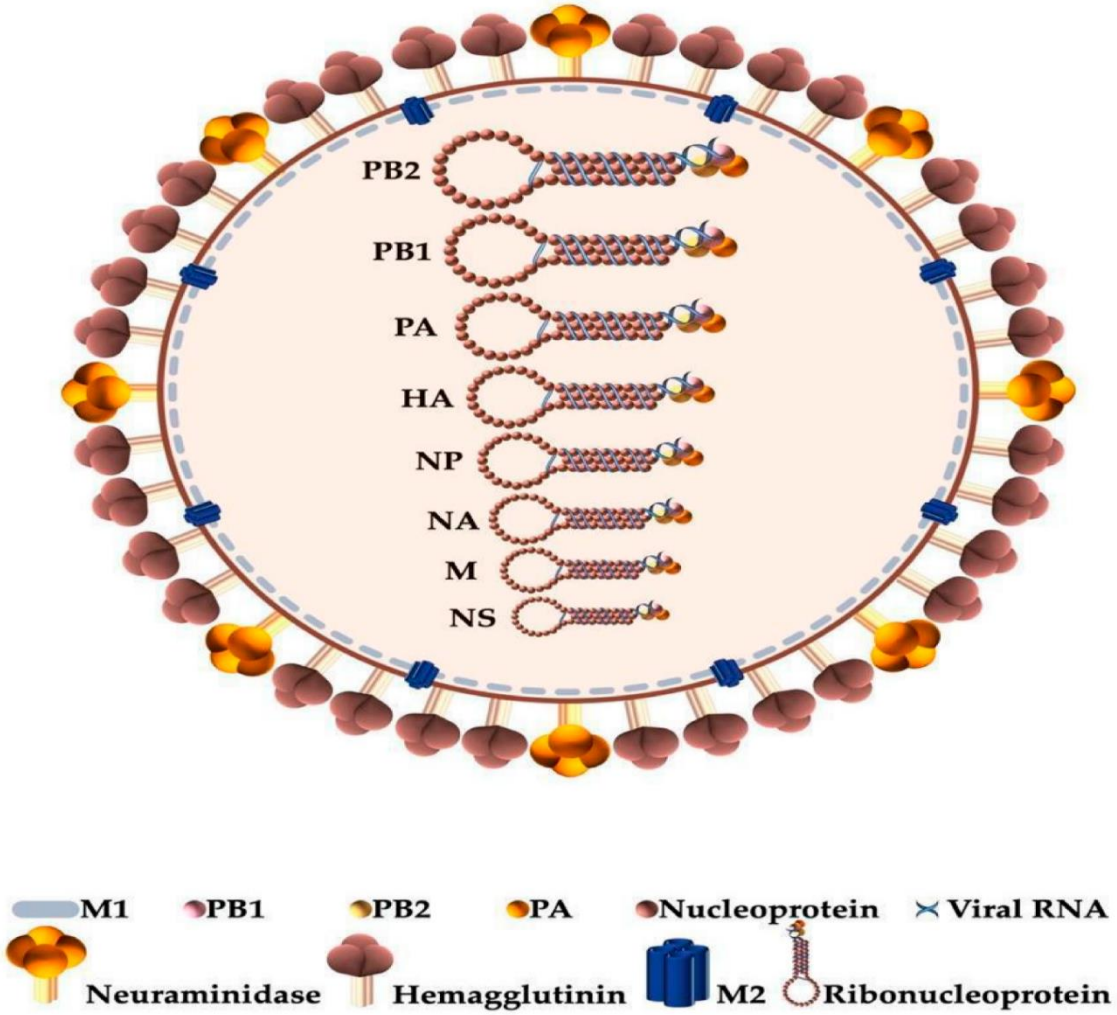


**Figure 1.1: Structure of influenza A virus and genome segments**
https://www.mdpi.com/1422-0067/21/4/1511/htm

## 1.3. Functions of influenza A gene segments

IAV is a genetically diverse virus that is classified using surface glycoproteins, e.g. the HA and NA proteins. The HA protein is critical in determining viral pathogenicity. It is synthesized as a precursor protein (HA0) and cleaved post-translationally by cellular serine proteases into two functional protein subunits, HA1 and HA2. Proteolytic cleavage of the HA protein is required for virus infection, with the outcome dependent on the host

and viral strain. The presence or absence of multiple basic amino acids at the cleavage site of HA protein influences IAV pathogenicity in a host, e.g. the insertion of a basic amino acid at the cleavage site may result from mutations at the IAV cleavage site. In addition, existing proteases, like furin, can cleave such mutated HAs, allowing the virus to spread. It is through this mechanism that a low pathogenic influenza virus becomes a high pathogenic influenza virus (Munch et al., 2001, Arbeitskreis Blut, 2009, Yamauchi, 2018). The NA protein efficiently releases virus progeny and allows them to spread to other cell targets. This protein helps the virus enter host cells by accelerating the breakdown of sialic acids connected to decoy receptors like mucins. It does this by decreasing virion aggregation and impeding viral binding through the HA. The functions of NA, as well as HA, involve interaction with sialic acid, which is expressed by glycoproteins at the cell surface (McAuley et al., 2019).

The PB2 protein is encoded by the longest genome segment and contributes to the infection cycle by participating in viral transcription and replication in the infected cell's nucleus. The nuclear localization signal targets the gene for transportation into infected cells' nuclei for viral transcription and replication. The protein is also responsible for the formation of the cap structure for viral mRNA and it has endonuclease activity and generates cap primers for viral mRNA synthesis using host mRNA (Cheung and Poon, 2007, Hao et al., 2019). The PB1 gene and the PB1-F2 gene is encoded by the second genome segment. The PB1 protein induces apoptosis through the interaction with mitochondrial proteins and there is an interaction between PB1-F2 and PB1 protein to retain the ribonucleoprotein. Photochemical cross-linking assays revealed that both substrate elongated product and viral RNA template were cross-linked at PB1, thereby identifying these proteins as components of the RNA polymerase. The PB1 subunit is essential for the assembly of the three polymerase protein subunits, as well as the catalytic function of RNA polymerization. This protein segment also contains an independent binding site for PA and PB2 (Cheung and Poon, 2007, Yamauchi, 2018). The PA protein is encoded by the third genome segment and contains nuclear localization signals required for transport into the nucleus. The PA subunit of influenza virus A is known to be essential for viral transcription and replication, and mutations near the carboxyl terminus inhibit transcription.

NP is encoded by the fifth segment and an RNA binding domain is found at the amino acid terminus of the protein. As a result, it's been suggested that the NP encapsulates the viral RNA in an *ad hoc* manner. It is not assumed that separation of the NP from the RNA template is essential for viral transcription and replication because the NP only

binds to the viral RNA backbone. As a result, NP may be involved in RNA polymerase's "switching" from transcription to replication. The NP has also been linked to vRNA nuclear transport, as it is a shuttle protein with nuclear localization signals. NP is thought to mediate the transport of incoming vRNPs from the viral particle into the nucleus during the early stages of viral infection. In contrast, progeny viral RNA's associated with NP, M1, and NS2 are exported to the cytoplasm for viral packaging in the late stage of infection (Cheung and Poon, 2007). Segment 7 encodes the M1 and M2 proteins that play multiple roles in the IAV cycle. The M1 segment is a collinear virion structural component with multifunctional proteins and plays a role in the replication process. According to reports, the M1 protein serves several functions for the virus, such as binding to RNA in a nonspecific manner and inhibits viral transcription and carries a nuclear localization signal which is considered to control vRNP nuclear transport. In addition, this protein promotes vRNP nuclear export and inhibits vRNP nuclear import (Cheung and Poon, 2007, Chen et al., 2013, Hao et al., 2019). The amino acid residues histidine and tryptophan are found in the transmembrane domain of the M2 protein and are responsible for pH regulation via ion channel activity. Furthermore, M2 ion activity has been reported to maintain the pH in Golgi vesicles to stabilize the native conformation of newly synthesized HA during intracellular transport and virus assembly (Cheung and Poon, 2007).

Segment eight is the smallest and encodes two proteins, NS1 and NS2. The NS1 protein is encoded in a collinear transcription gene segment that hinders transcription factors and interferon-stimulated genes from being effectively increased during interferon-induced antiviral host cell responses. By promoting viral mRNA translation, the protein enhances viral expression even further. The NS1 protein has two alleles, A and B, with A being found in both avian and mammalian species and B found only in avian species. Furthermore, the potential of different IAVs' NS1 proteins to interfere with the host immune response differs (Abolnik et al., 2007, Abolnik et al., 2016, Yamauchi, 2018). Prior studies indicate that the NS2 protein contributes to the normal transcription and replication of genomic RNAs via an unknown mechanism (Cheung and Poon, 2007).

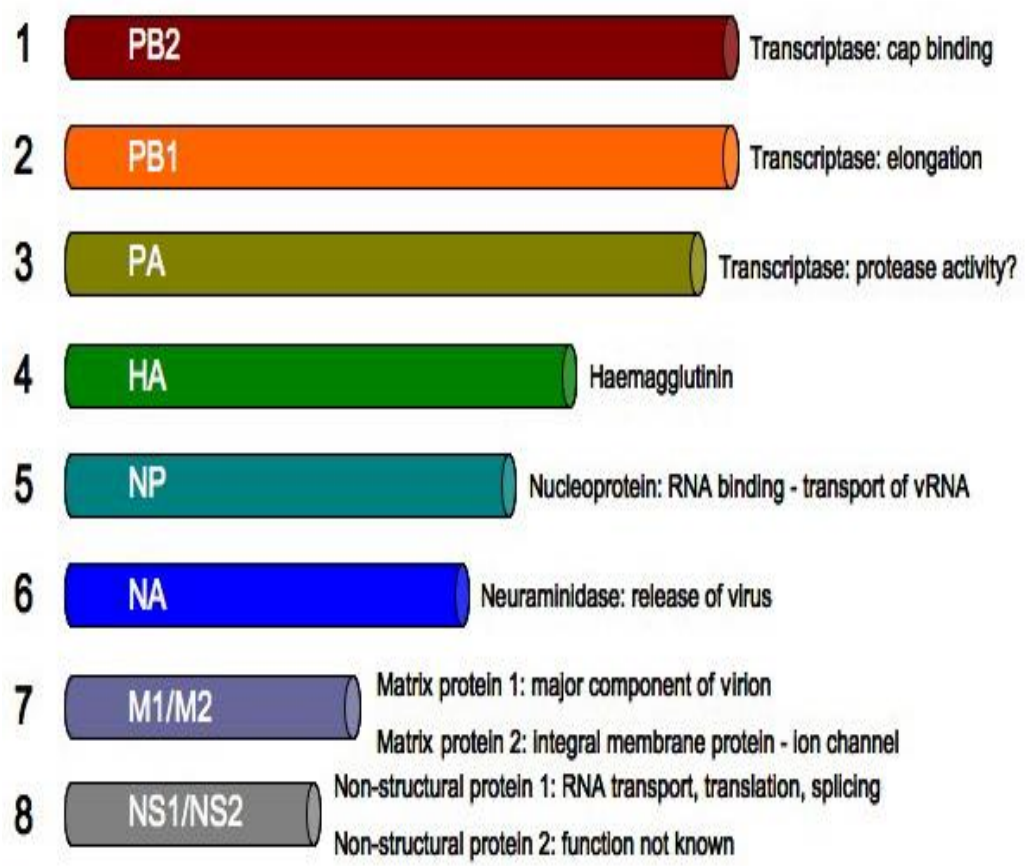


**Figure 1.2: Influenza A virus genome segments and brief description of their function** https://microbewiki.kenyon.edu/index.php/The_Threat_of_Airborne_Transmission_of_Influenza_A:_H5N1.

## 1.4. Ecology and Epidemiology

Avian influenza virus (AIV) is highly contagious and has been identified and isolated in over 100 birds from 26 different families. The virus infects a wide diversity of wild and domestic birds, however, wild birds are the reservoir of the virus, primarily waterfowl. Waterfowl species such as the *Anseriformes* (duck, geese and swans) and *Charadriiformes* (shorebirds and gulls) harbour H1 to H16 and N1 to N9 subtypes except for H17 and H18 which were recently discovered in bats. Combined, it leads to 144 estimated HA and NA subtypes (Webster et al., 1992, Joseph et al., 2017, Torrontegi et al., 2019).

AIV is most frequently found in *Anseriformes* and *Charadriiformes* species. These birds are globally distributed and are considered long-distance migrators and transmitters of the virus (Klenk et al., 2008). Both the *Anseriformes* and *Charadriiformes* play a key role in the epidemiology of AIV, however, the incidence of infections is mostly associated with

*Anseriformes* (dabbling ducks, mallard, pintails and teal) whereas *Charadriiformes* have a lower infection rate. Infection with low pathogenic avian influenza (LPAI) in waterfowl causes minimal signs of disease and these birds maintain the diverse subtypes of LPAI viral strains (an Dijk et al., 2018, Wahlgren, 2011). Different subtypes of LPAI and HPAI infect a wide variety of host's species including animals (swine and horses) humans and a wide variety of avian species (chicken, turkey and ostriches) (Figure 1.3) (Perkins and Swayne, 2002, Wahlgren, 2011).

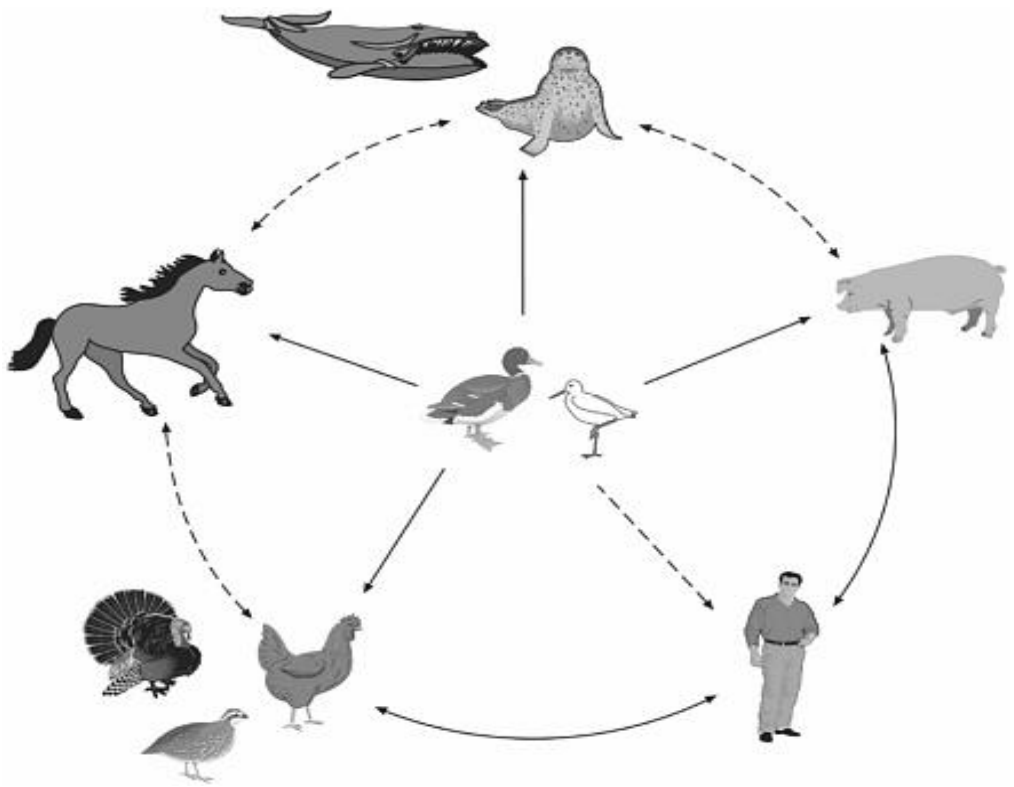

**Figure 1.3: Avian influenza virus spread from the natural host to different host species** https://journals.asm.org/doi/10.1128/JVI.00980-13

Interactions between *Anseriformes*, *Charadriiformes*, and *Gallinaceous* species frequently result in the direct and indirect introduction of AI viral strains into domesticated birds (Bergervoet et al., 2019). Waterfowl obtain AIV from the environment, such as contaminated food and shared water sources. The virus replicates in the cell lining of the gastrointestinal and respiratory tract of the host, thus AIV is asymptomatic in waterfowl and symptomatic in domestic birds (Webster et al., 1992). The prevalence of AIV in wild birds varies according to species, age, season, and geographic location. Because of the migration patterns of wild waterfowls, AIV can be carried over long distances. During their stopovers, these migrating waterfowl congregate to rest, breed, and forage with other species at a suitable wintering site, resulting in high local densities of the bird population and allowing virus transmission that can be distributed to susceptible host populations around the world (Klenk et al., 2008, Bergervoet et al., 2019). Waterfowl-to-poultry transmission can cause outbreaks of LPAI that trigger the development of the high pathogenic avian influenza virus (HPAI), or HPAI can be transmitted directly from wild birds to poultry (Webster et al., 1992, Arbeitskreis Blut, 2009).

## 1.5. Threat to poultry

A variety of AI viruses infect both wild and domestic birds and are commonly classified into two groups: LPAI and HPAI. Globally, influenza A viruses pose serious threats to poultry, especially the HPAI virus, which causes health problems such as lower egg production, mild respiratory disease and central nervous system impairment, which can ultimately result in death. There are only two subtypes of AIV that can acquire highly pathogenicity H5 and H7 (Webster et al., 1992, Kaoud et al., 2014, Venkatesh et al., 2018).

HPAI H5N1 is endemic in poultry in some countries, and this subtype has undergone a significant diversification over the past years. The H5 subtype has subsequently been isolated in many countries from different continents, such as Asia, Africa, and Europe causing outbreaks of infections in both poultry and wild birds and having a sporadic zoonotic transmission to humans thus raising a pandemic concern (Liu et al., 2016, Nagarajan et al., 2012).

AIV infections can have substantial effects on animal health and may cause significant financial losses to the poultry industry because of outbreaks. New influenza virus strains and subtype combinations may emerge as a result of AIV's rapid and unpredictable evolution. Changes in the virus genetic material can cause changes in the virus's features, such as enhanced virulence or a wider host range, as well as variants that are more likely to infect poultry. The recent outbreak of AIV in chicken emphasizes the necessity of global surveillance efforts for early detection and response (Bergervoet et al., 2019).

## 1.6. AIV threat in South Africa

In 1961, the first HPAI outbreak in South Africa was discovered in Common Terns (*Sterna hirundo*), with HPAI H5N3 being the virus strain. A total of 1300 birds died because of this virus. Waterfowl species (ducks and geese) were later discovered to be the principal hosts of LPAI presursors to HPAI viruses that emerge in poultry (Krauss and Webster, 2010, Verhagen et al., 2012).

HPAI H5N2 outbreaks occurred in South Africa in 2004 and 2006 on ostrich farms in the Eastern and Western Cape Provinces (van Helden et al., 2016). The LPAI precursor viruses originated in wild waterfowl populations, resulting in the loss of 30 000 ostriches through control measures, which represented 40% of all ostriches in the country at the time. Several other LPAI viruses have also been isolated in the same region over the past few decades, including H7N1 (1991), H5N9 (1994), H9N2 (1995), H6N8 (1998), and H10N1 (2001) (Banks et al., 2000, Abolnik et al., 2007). However, in a different region in South Africa, there was an outbreak of H6N2 in commercial poultry. The outbreak occurred in KwaZulu Natal (KZN) in June 2002 but the infection was traced back to ostriches as the source of the outbreak (Abolnik et al., 2007).

Ostriches (*Struthio camelus*) raised using an inclusive (free-range) environment are frequently exposed to waterfowl, including ducks and Egyptian geese (*Alopochen aegyticus*). There is evidence that epidemics of AIV strains originate from LPAI precursors in the wild duck reservoirs, resulting in substantial economic damage to the ostrich product industry (Abolnik et al., 2016).

## 1.7. Surveillance for AIV

Globally the surveillance of influenza A virus has been conducted since 1952, through the World Health Organization (WHO) surveillance and response system (Badar et al., 2013). Wild birds have been studied for the presence of AI viruses since the first identification of IAV in wild birds in 1961 (A/tern/South Africa/1961) (Verhagen et al., 2012). However, following the emergence of HPAI H5N1 viruses in Southeast Asia and the identification of the viral strain in migratory wild birds in 2005, wild bird sampling activities were increased. The growth in wild bird sampling initiatives around the world has increased the number of *Anseriformes* and *Charadriiformes* sampled (Verhagen et al., 2012).

Over the past 10 years, HPAI outbreaks have increased significantly. This spike is part of a long-term trend that shows five outbreaks for the 20 years 1959 to 1978, 13 outbreaks from 1979 to 1998, while nine outbreaks have occurred for the past 8 years (Pasick, 2008). A surveillance study conducted in the United States of America (USA) in 2006 revealed that certain HA gene sequences representing H4, H8, H10, H11, and H12 subtypes have no evidence of inter-continental exchange, whereas lower pathogenic AIV subtypes H1, H2, H6, H9, H13, and H16 have an inter-continental exchange between North America and Eurasian lineages (Deng et al., 2013).

Domestic ducks serve as a link between wild waterfowl and poultry, and they play an important role in viral ecology. Several AIV subtypes were detected in ducks, including the H5N1 and H9N2 virus strains, and are considered a public health threat. Although domesticated ducks can carry HPAI H5N1 variants and remain healthy, they can potentially succumb to the viruses in particular circumstances. In addition, in the 1970s and 1990s, the H9N2 subtype was detected in healthy ducks in Hong Kong, and it has subsequently expanded its host range to encompass poultry, becoming endemic in these birds. By transmitting genes to other influenza viruses, the H9N2 virus may pose a threat to human health (Krauss and Webster, 2010, Bergervoet et al., 2019, Tsai et al., 2020).

Venkatesh et al. (2018) discovered that other than *Anseriformes* ducks, Eurasian dabbling ducks were frequently infected with almost all IAV subtypes, but H13 and H16 was restricted to gulls. IA viruses in ducks are more prevalent during the autumn post-moult aggregation and migration stopover period with an average of 6.3 %, however, lower levels were observed in other post-moults in the northern Eurasian regions. A study conducted in Canada revealed that in wild ducks all 16 NA and 9 NA subtypes were

isolated except for H13, H14, H15 and H16, furthermore the IAV was isolated annually from both ducks and shorebirds (Krauss and Webster, 2010).

The IAV has a distinct seasonal pattern in temperate areas, with seasonal peaks during the winter. Tropical and subtropical regions experience seasonal variation in disease incidence due to mild winters, which are sometimes linked to the rainy season. In tropical and subtropical regions, the seasonal pattern is weaker (Badar et al., 2013). Eurasian waterfowls converge and mix with a varied range of Afro-tropical waterfowls in the Sub-Saharan region during the breeding season. AIV has been isolated in wild ducks during their wintering grounds in Europe, North America, and Africa, with LPAI discovered and isolated from many large wetlands holding diverse species in these regions. This revealed that the Afro tropical regions' environmental circumstances are favourable for AI persistence and spread of the virus (Gaidet et al., 2012). Environmental samples such as faeces, water, and wetland sediments, have become increasingly popular for AIV surveillance. Waterfowl can densely populate wetlands because they provide vital refuge locations during migration. Wetland areas contain a diversity of AIVs from numerous avian hosts since wild migratory ducks naturally harbour and excrete considerable volumes of AIV in their faeces for extended periods (6–28 days). As a result, wetlands are important places for AIV surveillance based on sediment and excreted faeces (Tindale et al., 2020).

Previously the surveillance of AIV was mainly focused on detecting and eliminating HPAI infections in poultry, but since LPAI H5 and H7 subtypes can undergo mutation and gain high pathogenicity the detection and control of these viruses have become mandatory (Comin et al., 2012, Moriguchi et al., 2021). Passive surveillance was used to identify HPAI early on because the virus causes obvious clinical symptoms and significant mortality in most poultry species. However, because there are no obvious symptoms of LPAI and mortality is low, active surveillance is used. The effectiveness of LPAI surveillance programs has primarily been assessed in terms of locating infected birds or assessing their risk of infection, rather than their suitability as an early warning system for new introductions (Comin et al., 2012).

## 1.8. Problem Statement

Studies have previously prioritized sampling waterfowl for IAVs directly rather than the wetland areas they inhabit, where a high concentration of IAVs are excreted (Hood et al., 2021, Ramey et al., 2022) The wetland habitat provides a social and ecological platform for waterfowl, migratory birds and wild birds (Kim et al., 2019). The majority of emerging epizootic and zoonotic diseases result from the interface of wetland habitat and human-dominated landscapes (such as farmlands) (Pérez-Ramírez et al., 2010, Wu et al., 2020). Researchers have shown that backyard farms near wetlands are vulnerable to IA viral transmission (Kaoud et al., 2014). The reservoir hosts (migratory birds and waterfowl) have multiple combinations of HA and NA proteins, thus these birds allow IAVs to spread over long distances resulting in the introduction of new viruses to naive bird populations (Venkatesh et al., 2018, Hopken et al., 2020, Ramey et al., 2022).

There have been few studies that have reported the isolation of IAV from environmental faecal samples collected directly from the wetland habitat and the recovery and isolation of viable IAVs from faeces deposited in the environment. The identification of IAVs from environmental faeces may provide necessary information for the circulating IAVs in waterfowl communities in a wetland habitat within a human-dominated area. In addition, the research study will improve the IAV genome sequence identification from environmental faecal samples.

## 1.9. Research questions

- Are faecal samples sufficient to detect the presence of IAV in wild birds and also identify the genome segments of the virus?
- Do local habitats preserve IAV excreted by wildbirds and various bird species?
- Can pre-treatment methods remove inhibitors from faecal samples and improve recovery of the virus?

## 1.10. Research Aims and Objectives

The aim of the research study was to improve the detection of influenza A virus from wild bird faecal samples obtained from a site in Pretoria, South Africa. The objectives of the research study were as follows:

### Optimize the full genome RT-PCR for influenza A virus (Chapter 2)

The objectives of this part of the study were:

- To use prepare a spiked duck faecal sample with which to optimize the full genome M-RTPCR
- To optimize theprimer set used in the M-RTPCR
- To optimize the $MgSO_4$ concentration
- To optimize the nucleic acid volume and annealing temperature of the M-RTPCR
- To optimize the M-RTPCR using Phusion flash high fidelity kit (Life Technologies)

### Field samples screening and IAV genome application (Chapter 3)

The objectives of this part of the study were to perform:

- Screening of faecal samples collected from wild birds at the study site
- Pre-treatment methods to improve recovery of viral nucleic acid and reduce inhibitors from faecal samples

### Identification of positive IAV samples using Next Generation Sequencing (Chapter 4)

The objectives of this part of the study were to:

- Construct an IAV reference subtype sequence database
- Identify the subtype through an assembly-to-reference approach
- Perform verification of sequence identities through BLAST analysis

Approvals for this study were obtained from the UP Animal and Research Ethics Committee under project no. REC032-19 and from the Department of Agriculture, Land Reform and Rural Development under Section 20 permit no. 12/11/1/1/8 (1217) (Addendum).

# CHAPTER 2: OPTIMIZATION OF A FULL GENOME RT-PCR FOR INFLUENZA A VIRUS

## 2.1. Introduction

Previously virus isolation in embryonated specific-pathogen-free (SPF) eggs were considered a standard technique for the detection and characterization of IAV. However, this method is time-consuming, costly, and has a high risk of contamination from biological matter (Dhumpa et al., 2011, Elizalde et al., 2014). Since the establishment of molecular methods, characterization and identification of IAV has been revolutionized and become significantly more efficient, thus the most frequently used methods include real-time RT-PCR (rRT-PCR) and multisegmented reverse transcription-PCR (M-RT-PCR). rRT-PCR has increased sensitivity, specificity, and speed when compared to traditional methods. This technique is frequently employed for targeting the matrix (M) gene with a detection limit of 0.1 $EID_{50}$ to $10^{-3}$ copies to confirm the presence of IAV, and HA and NA gene based on PCR. It can further be used to characterize the different IAV subtypes (Lee and Suarez, 2004).

rRT-PCR assays can be used to detect viral RNA, resulting in high specificity and sensitivity when compared to conventional virus isolation (Lee and Suarez, 2004). Furthermore, this method was determined to have a high correlation with virus isolation but unlike virus isolation, it can test a large number of samples in a fast-paced and sensitive manner, thus increasing productivity and reducing the risk of cross-contamination. This technique is considered the dominant method because it is widely used as a tool for virus isolates in most diagnostic reference laboratories and is also used for screening IAV in field samples (Lee and Suarez, 2004).

Zhou et al., 2009 initially reported a set of universal primers for full amplification of the IAV genome, which utilized numerous segment-specific primers to generate full complementary DNA lengths, and additionally applied a single reaction to amplify all eight segments of IAV (Zou et al., 2016). The approach of full genome amplification is to reverse transcribe and amplify the RNA genome, using primers corresponding to the conserved 12 nucleotides at the 3' terminal and 13 nucleotides at the 5' terminal of the viral RNA, thus permitting detection of all eight genome segments in a single reaction (Fouchier et al., 2000, Ellis and Zambon, 2002, Zou et al., 2016).

The amplification of influenza genome segments is a vital step to enrich the entire genome of IAV preceding Next-generation Sequencing (NGS) (Zhou et al., 2009, Zou et al., 2016). The method of Zhou et al, (2009) for IAV has been used for genome PCR with significant success to amplify a full genome from clinical samples such as tracheal swabs and tissues at the University of Pretoria but less successfully with faecal and cloacal swabs (Abolnik et al., 2019). The presence of IAV excreted into the environment contains PCR inhibitors and bacterial contamination, therefore using environment sampling instead of catching live birds also reduces the disturbance of wildlife (Borrelli et al., 2020).

The objective of this chapter was to use spiked faecal duck samples to optimise the efficiency of M-RTPCR full genome assay for the faecal samples by optimising different components such as primers, annealing temperature, the input of template, $MgSO_4$, and comparing kits to identify the most cost effective method.

## 2.2. Materials and Methods

### 2.2.1. Nucleic acid extraction

The total RNA of an isolated H6N2 virus (H44954/2016) in allantoic fluid from the University of Pretoria repository was extracted using Trizol® reagent (Invitrogen-Life Technologies). Trizol® reagent (750 µl) was added to a 1.5 ml Eppendorf tube with 250 µl of the allantoic and incubated for five minutes. Two hundred microliters of chloroform was added and the tube was vigorously shaken then incubated for 10 minutes. The mixture was centrifuged for 15 minutes at 13000 rpm (Sigma 1-14 microcentrifuge) the upper aqueous phase was transferred into a new Eppendorf tube. Six hundred microliters of Isopropyl alcohol was added to the tube briefly vortexed and incubated at room temperature for 10 minutes. The tube was centrifuged for 10 minutes discarded the supernatant subsequently the RNA pellet was washed with 700 µl of 70 % ethanol and centrifuged for 5 minutes. Ethanol was removed and the RNA pellet was allowed to air dry for 10 minutes, and then reconstituted with 50 µl of elution buffer (Whitehead Scientific).

### 2.2.2. Optimized primer set

Primers originally described by Zhou et al, (2009) were compared with a set of modified primers by Lee et al., 2013 (IDT-Whitehead scientific) (Table 2.1). The superscript ™ III One-Step PCR system with Platinum Taq High Fidelity DNA polymerase kit was used to carry out the reaction. The reverse-transcription reaction was carried out on an Applied Biosystems (ABI) Veriti Thermal cycler PCR instrument (Life Technologies), and the temperature parameters were as follows: 42°C for 60 min (Reverse transcription (RT), 94°C for 2 min (inactivation of RT enzyme and activation of Taq enzyme), then 5 cycles 94°C for 30 s (denaturation), 45°C for 30 s (annealing), and 68°C for 3 min (extension), followed by 31 cycles 94°C for 30 s respective second annealing temperature ($T_a$), 57°C for 30 s, and 68°C for 3 min (final extension). M-RTPCR products were subjected to electrophoresis on a 1% agarose gel (molecular grade-Celtic Molecular Diagnostic) stained with ethidium bromide (Life technologies) and visualized on an E-gel imager ™ System with UV Light Base (Thermo-Fischer Scientific-Life Technologies).

Table 2.1: Standard and optimized primer set of M-RTPCR

| Primer names | Primer pair sequence | Primers designed by |
|---|---|---|
| Reverse MBTuni 12 | 5'-ACGCGTGATCAGCAAAAGCAGG-'3 | Zhou et al.,(2009) |
| Reverse MBTuni 12 DEG | 5'GCGTGATCAGCRAAAGCAGG-'3 | Lee at al.,(2013) |
| Forward MBTuni13 | 5'-ACGCGTGATCAGTAGAAACAAGG-'3 | Zhou et al.,(2009) |
| Forward MBTuni13 DEG | 5'-ACGCGTGATCAGTCGAAACAAGG-3' | Lee et al.,(2013) |

* R = A or G nucleotide in the primer sequence

### 2.2.3. Optimization of $MgSO_4$ concentration

Different concentrations of $MgSO_4$ were added with volumes ranging from 2 mM to 5 mM and included RNase inhibitor (Invitrogen-Life Technologies). Nuclease activity is controlled by RNase inhibitors, which prevent DNA replication (Wilson, 1997). The reaction mixture consisted of the following components: Superscript ™ III One-step system with high fidelity kit (10 µl); primers ( 1 µl) each; $MgSO_4$ (2 µl; 4 µl; 6 µl; 8 µl; 10 µl; 12 µl and 14 µl) and RNase inhibitor (0.2 µl). The PCR products were subjected to 1 % agarose gel electrophoresis and visualized the amplicons on the E-gel imager (Life Technologies).

### 2.2.4. Optimization of M-RTPCR using an increased nucleic acid volume and different annealing reverse transcription temperature

To investigate whether increasing the RNA template will improve M-RTPCR results, the assays were performed using two sets of volumes and two pre-mixes. The first mixture contained RNA and primers and the second contained master mix reagents. On the first mixture, one tube had 5 µl of RNA while the other had 14 µl and added 1 µl each of primers then denatured at 95°C for 2 min (snap cool method). The second mixture prepared contained 2X reaction buffer (25 µl), Superscript RT-Platinum high fidelity Taq (1 µl), and $MgSO_4$ (8 µl). Mixture 1 was added to mixture 2 and put on the thermal cycler (Applied Biosystem Veriti-Life Technologies) following these condition parameters: 55° for 60 min (RT); 94° for 2 min followed by 5 cycles of extension of 94° for 30 sec; 45° for 30 s and 68° for 4 min, 31 cycles of 94° for 30 s; 57° for 30 sec; 68° for 4 min and the final stage of 68° for 5 min and a final hold at 4°. The thermal amplification profile

was the same as previously described, except for the change in the reverse transcription and snap cool method before the run.

### 2.2.5. Optimization of M-RTPCR using Phusion flash high fidelity kit

The original method described by Zhou et al., (2009) using a Superscript III PCR system with Platinum Taq high fidelity DNA pol kit was altered by employing a lower-cost kit (Table 2.2), namely Phusion flash high fidelity (Life Technologies). The reaction mixture consisted of Phusion flash high fidelity PCR master mix (10 µl); modified primers (1 µl) of 10 pmols each; M-MLV reverse transcriptase (200U/µl) (0.5 µl) (Life Technologies); RNase inhibitor (0.2 µl); and nuclease-free water (2.3 µl). The temperature cycling conditions were modified from the initially described conditions by Zhou et al., (2009) and in 2.2.4. Thermal cycle conditions were as follows: denaturation at 37° for 20 min; 98° for 10 s; 35 cycles of annealing temperature at 98° for 5 s 58° for 15 s; 72° for 2 min and extension at 72° for 4 min.

Table 2.2: Comparative costs for M-RTPCR reagents

| Reagents | Reagents | Unit size | Total vol p/ k (µl) | Cost (R) Vat Incl | Number of test reactions per kit | Vol per run (µl) |
|---|---|---|---|---|---|---|
| SuperScript III RT/ Platinum High fidelity mix | Superscript III RT/PCR platinum Taq Mix | 100 | 100 | 11 010.0 | 50 | 2 |
| | Magnesium Sulfate($MgSO_4$) (5mM) | | 500 | | 50 | 10 |
| | 2X Reaction Mix (X3) | | 1000 | | 40 | 25 |
| **Total per reaction** | **R751.65** | | | | | |
| Phusion Flash high fidelity PCR | Phusion flash high fidelity master mix( 2X) | 500 | 1000 | 4010.00 | 100 | 10 |
| | M-MLV reverse transcriptase Taq | 40 000 | 200 | 4510.00 | 400 | 0.5 |
| | RNase inhibitor | 2000 | 100 | 1480.00 | 500 | 0.2 |
| **Total per reaction** | **R54.34** | | | | | |

### 2.2.6. Preparation of a duck faecal sample spiked with IAV

A fresh faecal duck sample was collected from the dam embankment at the African Pride Irene Country Lodge. The faecal duck sample was screened for presence of IAV and the negative sample was proceeded to be weighed and diluted. Five grams were weighed off and then added to 20 ml of distilled water and vortexed to create a slurry. Two millimetres of the slurry was transferred to a 5 ml Eppendorf tube and 2 ml of LPAI H6N2 allantoic ($EID_{50}10^{6.8}$) fluid was added and thoroughly mixed. In separate tubes, 2 ml of viral transport media (VTM) was pipetted to each tube labelled 1:2 to 1:1024 for a two-fold dilution factor. The VTM consisted of brain-heart infusion broth (BHI) (Sigma Aldrich) 10% glycerol and antibiotics (penicillin-streptomycin (1ml) (Sigma Aldrich) enrofloxacin ((1ml) Bayer Corporation) and doxycycline (100 mg/ml) (Pfizer). The spiked faecal solution was then subjected to a two-fold serial dilution from 1:2 to 1:1024. RNA was extracted as described above.

### 2.2.7. Sensitivity of rRT-PCR assay on serially diluted spiked faeces

To determine the detection limit of the serially diluted viral RNA, the RNA extracted from spiked duck faeces was screened for the Matrix (M) gene of IAV. The Vetmax™ Plus One-Step RT-PCR Kit (Life Technologies) was used on an automated Applied Biosystems Step OnePlus instrument (Life Technologies). The primers and probes for the M gene are those described by Spackman et al., (2003). The thermal cycling parameters were as follows: 48°C for 10 min; 1 cycle of 95°C for 10 min; 40 cycles of 95°C for 15 s and 53°C for 45 s. The same RNA was then tested using the optimized M-RTPCR as described in 2.2.5.

## 2.3. Results

### 2.3.1 Comparison of standard and optimized primer set

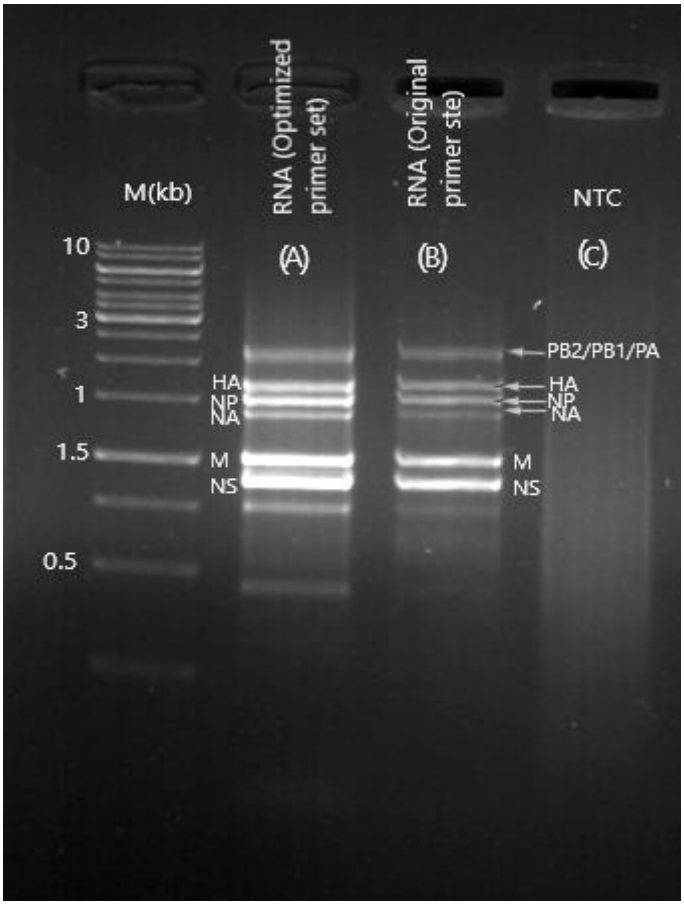


**Figure 2.3.1 Comparison of M-RTPCR using different primer sets. Lane A consisted of optimized primers described by Lee et. al., (2013) and Lane B consisted of original primers described by Zhou et.al.,(2009) and the last lane C contained no template control (NTC). The 1kb ready to use GeneRuler (Life technologies) was used to measure the amplified genome segments.**

Figure 2.3.1 shows 5 µl of the amplified PCR products, which were loaded into the gel. Both primers produced bands for all eight genome segments, but the optimized primers by Lee et al., (2013) were able retrieve more genetic material of the IAV, thus producing well defined visible bright genome DNA bands.

### 2.3.2. Effects of different $MgSO_4$ concentration

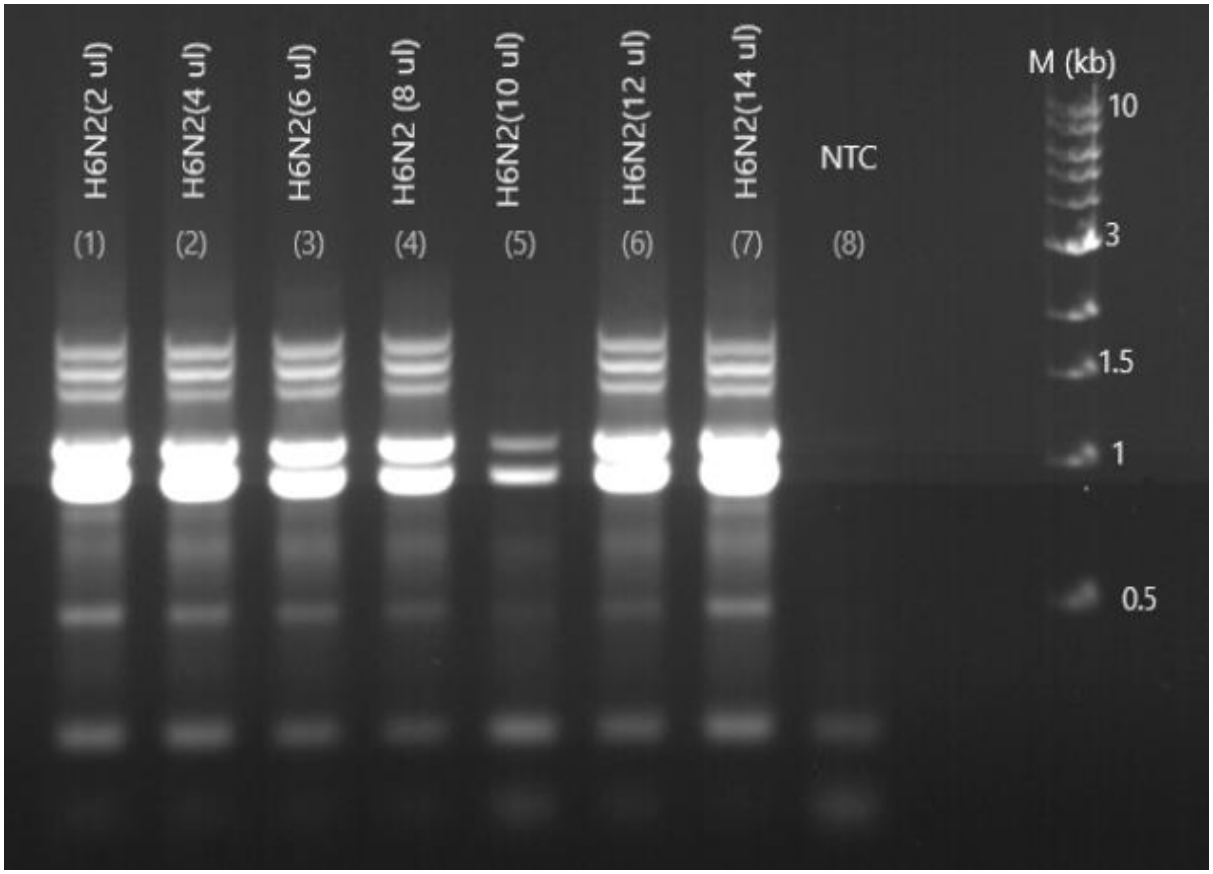


**Figure 2.3.2: Amplification of the viral genome segments using different concentrations of $MgSO_4$. The volumes added were as follows from lane 1 to 8 respectively: 2 µl, 4 µl; 6 µl; 8 ul; 10 µl; 12 µl and 14 µl, the last lane contained the no template control (NTC).**

Figure 2.3.2 shows that $MgSO_4$ had little effect on PCR efficiency. Only six of the reactions were able to produce amplification of the genome segments, for lane 5 only two gene segments were amplified. This could have resulted from incorrect volume pipetted in the reaction tube or possible evaporation due to the tube not being sealed properly.

### 2.3.3. Effects of RNA template volume and annealing temperature

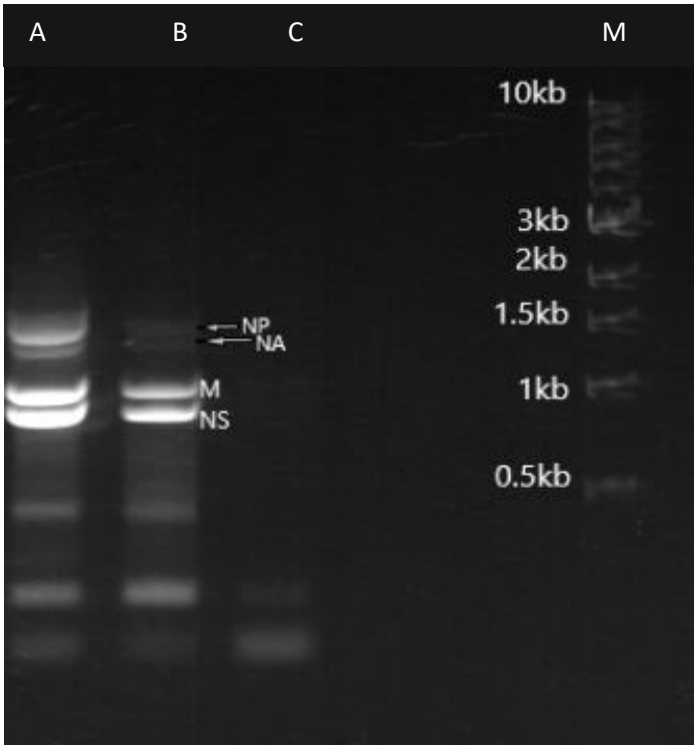


**Figure 2.3.3: Comparison of different template volumes and annealing temperatures. Lane A consists of an RNA template with 5 µl volume, lane B consists of an RNA template with 14 µl volume.**

Fig 2.3.3 shows amplification of the genome segments on both lanes from the snap cool method, but Lane A had bands that were more visible than and defined than Lane B. An RNA template of 5 µl volume extracted from faecal material was more suitable to use for full genome amplification.

### 2.3.4. Comparison of the efficiency of Superscript III kit and Phusion flash mix

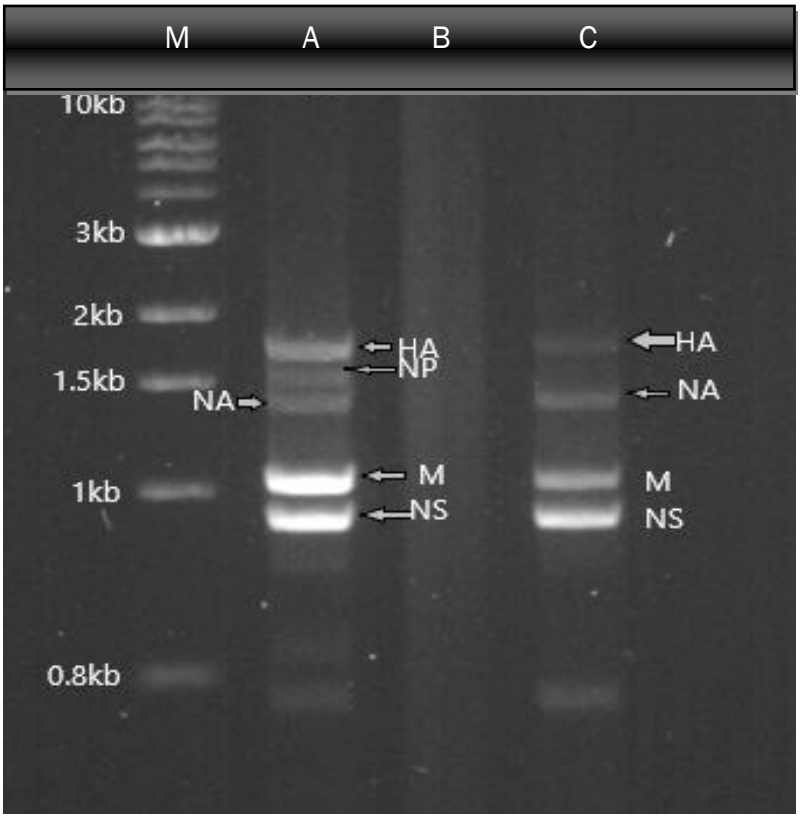


**Figure 2.3.4: Agarose gel electrophoresis results obtained from comparison of the Phusion flash mix in Lane A and Superscript III kit in lane C. Lanes A and C had an RNA template volume of 5 µl and lane B contained the NTC template.**

Figure 2.3.4 shows the genome segments amplified by both Superscript III kit and Phusion flash mix. However, the Phusion flash method was able to amplify more genome segments and was more visible. Whilst the superscript method amplified fewer genome segments with the HA and NA obtained faint bands.

Based on the overall results obtained, the RNA template from the faecal sample was amplified using different methods with each different optimization approach the template amplified the higher and lower molecular weight genome segment especially the HA and NA which are often used to determine the subtype of IAV. The last approach shows that the Phusion flash mix which is of a lower cost is capable of amplifying the full genome segment from the RNA template.'

## 2.3.5. Sensitivity of rRT-PCR assay on serially diluted spiked duck faeces

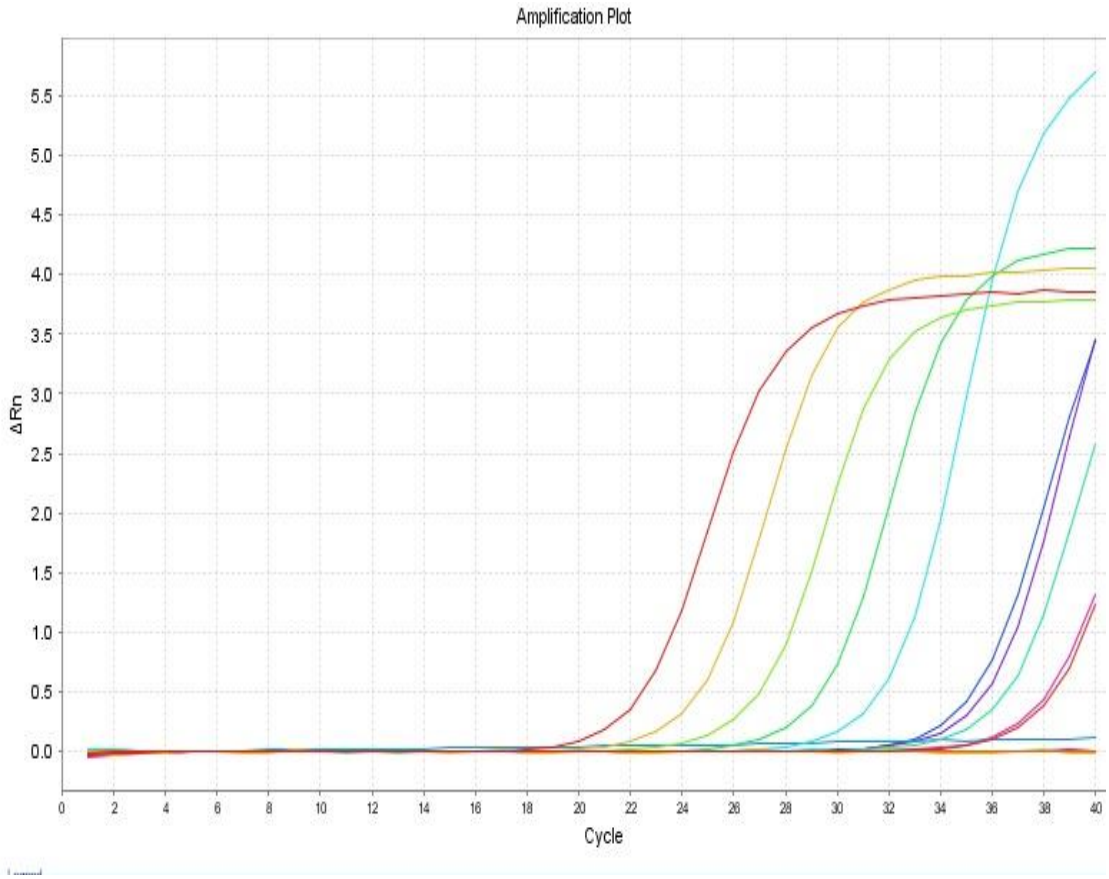


**Figure 2.3.5: rRT-PCR amplification of an RNA extracted from a serial dilution of a spiked duck faecal sample.**

Figure 2.3.5 shows the amplification and sensitivity of the serial dilution factor obtained from a spiked duck faecal sample spiked with viral RNA. The rRT-PCR assay was conducted to detect the presence of the IA viral RNA copies and determine the cut off cycle threshold (Ct) value of the serial dilution factor from the spiked faecal samples. The Ct values of the serial dilutions were obtained and tabulated.

Table 2.-3 : Sensitivity evaluation for the serial dilution factor

| Dilution Factor | Ct Value |
|---|---|
| 1:2 | 21.17 |
| 1:4 | 23.24 |
| 1:8 | 25.65 |
| 1:16 | 28 |
| 1:32 | 30.35 |
| 1:64 | 33.87 |
| 1:128 | 34.42 |
| 1:256 | 36.79 |
| 1:512 | 37.05 |
| 1:1024 | Undetermined |

Table 2.3 shows the cycle threshold (Ct) value of the dilution factor, and the first five dilutions (1:2-1:32) yielded a Ct value that was less than 31 indicating a higher presence of viral RNA copies. Four of the dilution factors had Ct's ranging from 33 to 37, which indicated low numbers of viral RNA copies. The final dilution factor was undetermined therefore indicating it was negative for the presence of viral RNA copies. Furthermore, the spiked serial dilution samples were subjected to the M-RTPCR assay to determine whether the genome segments could be amplified.

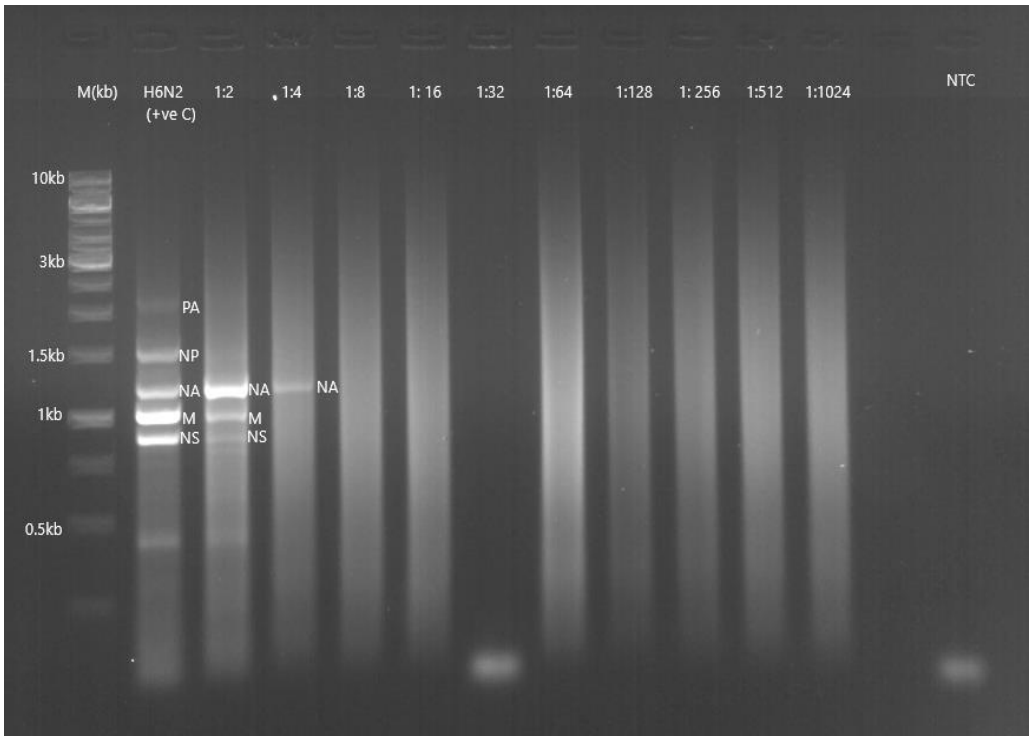


**Figure 2.3.6*:* Results of the use of the Phusion flash kit for the M-RTPCR on duck faecal samples spiked with viral RNA in serial dilution. Lane 1 is a 1kb ladder, Lane 2 is the undiluted spiked faecal sample and Lanes 3 to 12 are the dilutions, and Lane 14 is NTC.**

Serial dilutions of spiked faecal samples from section 2.3.1 to 2.3.4 were subjected to M-RTPCR using the optimized method. The results in Figure 2.3.6 above show the IAV genome-specific bands which are only visible in the undiluted spiked sample and the 1:2 dilution amplified three genome segments, whereas the 1:4 dilution had one genome segment NA that amplified. Dilution factors 1:8, 1:16, 1:128, 1:256, 1:512 and 1: 1024 had smears in their lanes, with no visible amplification of the genome segments, indicating possible non-specific amplification, but could mask IAV genome segments. Dilution 1:32 had no amplification which could have resulted possibly from handling errors during preparation.

## 2.4. Discussion

For this chapter, five parameters were optimized to maximise full genome amplification from faecal samples for M-RTPCR. Modified primers by Lee et al., (2013) were compared with the original primers by Zhou et al.,(2009). Modified primers were confirmed to be more sensitive under different conditions. The $MgSO_4$ concentration proved to be unimportant as different concentrations produced similar results. Furthermore, an increased RNA template of 14 µl showed poor amplification of the gene segments, especially the internal genome segments, producing faint and less visible bands. However in other clinical samples such as tracheal swabs and pooled organs the 14 µl volume worked well and amplified genome segments (Abolnik et al., 2019). The optimized M-RTPCR method using Phusion flash high fidelity master mix demonstrated the ability to amplify genome segments through the application of optimized thermal cycling conditions. The annealing temperature of 37°C for 20 min proved to be suitable for genome amplification when compared to the higher annealing temperature in the Superscript protocol of 57°C for 60 min. Based on overall findings for optimization, the Phusion flash kit was established to be cost-effective with a short test turnaround time than the Superscript III kit which makes it useful when handling large number of samples.

A serial dilution of a spiked faecal duck sample was tested using both rRT-PCR assay and optimized M-RTPCR. According to Dovas et al., (2010) concentrations of IAV in environmental faecal samples are often below the threshold of detection of most common diagnostic methods, but rRT-PCR assay is a reliable approach for detection and quantification of the virus. Firstly, RNA of the spiked duck faecal dilutions was tested by rRT-PCR; this assay amplifies small conserved regions usually M1/M2. The results obtained demonstrated IAV RNA could be detected in a 1:512 dilution with a Ct of 37.05. Clinical samples with a Ct of 33.7 and a virus titre of 25 copies /µl were able to achieve full genome through the M-RTPCR method. In addition, to the reduction of the virus to 5 copies/ µl the genome segments could be determined due to the complete coverage of the HA and NA genes (Zou et al., 2016). The results obtained from this chapter demonstrate the visible genome segments up to 1:4 dilution with a Ct of 23.24, therefore indicating that faecal swabs with Ct's ≤24 are best for full genome amplification. Lower Cts may only yield partial genome segments.

# CHAPTER 3: FIELD SAMPLE SCREENING AND IAV GENOME APPLICATION

## 3.1. Introduction

Influenza A virus (IAV) causes minimal to severe disease in domestic and wild birds which may result in high levels of mortality depending on the viral strain and host. Waterfowl such as ducks, geese, swans and shorebirds are considered the natural host of the virus and are a constant source of infection for domestic birds. Waterfowl are important hosts in the epidemiology of IAV, as indicated by prior studies of their predictable temporal and spatial patterns of infection (Dovas et al., 2010). According to Nazir et al. (2011), there is growing evidence that IAV transmission within waterfowl populations is heavily reliant on environmental persistence.

IAV spreads from wild birds to domestic birds through faecal (cloacal) and oral (oropharyngeal or tracheal) secretions. Because IAV viral infections are mucosal and replicate in the respiratory or digestive tract, oral and tracheal samples are frequently used to screen for the presence of IAV (Das et al., 2009; Pannwitz et al., 2009). Tracheal and cloacal swabs and tissues are considered to be optimal for the detection of IAV, however, obtaining these samples require capturing and handling wild birds. Field sampling such as the collection of fresh faeces is a convenient, non-invasive and cost-effective approach (Ramírez-Martínez et al., 2018).

One of the most common routes of spread of IAV in waterfowl is through faeces. In a previous study, infected birds were found to excrete more IAV in their faeces than in their nasal secretions (Torrontegi et al., 2019). Several factors affect the persistence of IAV in the environment, including local environmental conditions, for example, temperature, salinity and organic matter (Kurmi et al., 2013). Faecal samples excreted by wild waterfowl can survive and be preserved in the wetland environment at a low temperature of 28°C for five days and 4°C for eight weeks (Nazir et al., 2011, Ofula et al., 2013). Environmental persistence, according to Lickfett et al. (2018), may allow for short and long-term IAV maintenance by providing a mechanism for transmission between spatially or temporally separated bird populations, and environmental transmission is essential for infection maintenance. The collection of faecal samples allows the detection of IAV in live domestic birds, wild birds, and waterfowl, particularly migrating birds, which carry various strains of the virus and can be used to identify the undetected IAV strains

(Nourouzian and Vasfi Marandi, 2007). In addition to being an active surveillance effort for wild waterfowl, environmental faecal sample analysis could be useful in identifying IAV circulating in these birds (Pannwitz et al., 2009). Faecal samples, however contain a significant amount of contaminants such as , bacteria and other biological matter. According to Pawar et al., (2019) the background nucleic acid consisting of DNA from faecal swab samples could negatively affect IAV genome amplification.

The purpose of this chapter was to take swabs from fresh faecal samples from a mixed population of ducks and wild birds in a wetland habitat and and to screen them for thepresence of IAV using rRT-PCR. The complete genome segments were then amplified using the optimized M-RTPCR approach described in Chapter Two. In addition, pretreatment methods were compared to remove inhibitors from the faecal swab samples in order to improve genome amplification.

## 3.2. Materials and Method

### 3.2.1. Environmental sampling

A total of 2144 of fresh faecal swab samples were collected at African Pride Irene Country Lodge from a diverse population of wild birds and waterfowl that included mostly Egyptian geese (*Alopochen aegyptiacus*), Yellow-billed duck (*Anas undulata*), Black swan (*Cygnus atratus*), Red Knobbed coot (*Fulica cristata*), African Sacred Ibis (*Threskiornis aethiopicus*) and Hadeda Ibis *(Bostrychia hagedash)*. Samples were collected weekly for two months from January to February 2021. Samples were collected by inserting a sterile swab (Carlo Roth sterile applicator-Separations) into fresh faeces before placing each swab into 2 ml viral transport media (VTM) consisting of brain-heart infusion broth (BHI) (Sigma Aldrich), 10% glycerol, antibiotics (penicillin-streptomycin (1ml) (Sigma Aldrich) enrofloxacin (1ml) (Bayer Corporation) and doxycycline (100 mg/ml) (Pfizer). A styrofoam box with an ice pack was used to transport samples to the laboratory at the University of Pretoria and they were processed immediately.

### 3.2.2. Screening of IAV (M) gene by rRT-PCR

The IndiMag 48s automatic extraction equipment (Whitehead Scientific) and the IdiMag pathogen kit (Whitehead Scientific) were used to extract nucleic acid from faecal swabs according to the manufacturer's instructions. The extracted RNA was vortexed vigorously tested for the presence of IAV, using the Vetmax™ Plus One-Step RT-PCR kit (Life Technologies) with primers and probes described by Spackman et al. (2003). The rRT-PCR assay was performed on the Step One plus instrument (Life Technologies) using the optimal thermal temperature parameters described in Chapter 2.

### 3.2.3: Testing of positive faecal swab samples using optimized M-RTPCR

Positive faecal swabs with a Ct value less than 34 were subjected to optimized M-RTPCR. The reactions contained a Phusion flash high fidelity PCR master mix (10 µl); modified primers (1 µl) each; M-MLV reverse transcriptase (200U/ µl) (0.5 µl) (Life Technologies); RNase inhibitor (0.2 µl); nuclease-free water (2.3 µl) and 5 µl RNA template. Thermal temperature parameters are those described in Chapter 2.

### 3.2.4. Pre-treatment methods of spiked duck faecal samples and comparison of extraction methods

#### *3.2.4.1. Addition of RNA later ™*

The spiked faecal serial dilution with a volume of 2 ml was treated by adding the same volume of RNA later ™ (Invitrogen-Life technologies) and vortexed vigorously for 10 sec and refrigerated overnight at 4$^{o}$C. The RNA was extracted using the standard Trizol method described in Chapter 2 and IndiMag 48s (Whitehead Scientific), then screened for the matrix gene using rRT-PCR assay.

#### *3.2.4.2. Filtration of samples before extraction*

The serial dilution of the spiked faecal sample was treated by filtering each sample with a 0.45 µm filter (Lasec) into a new sterile 5 ml Eppendorf tube. The filtered samples were further extracted manually using Trizol method and automatically using the IndiMag 48s instrument (Whitehead Scientific).

#### *3.2.4.3. Optimizing of Trizol extraction method using Phenol-Chloroform Isoamyl*

The Trizol standard method was optimized by substituting the conventional chloroform with the PCI (25:24:1v/v-Invitrogen-Life technologies). The optimized protocol steps are as follows:

i. 200 µl of faecal solution and 750 µl of Trizol reagent were added to an Eppendorf tube and incubated at room temperature (RT) for 5 min.
ii. Phenol: chloroform: isoamyl (PCI) alcohol (200 µl) was added into the solution and vortexed for ten seconds then kept at RT for 10 min before centrifugation at 13 000 rpm 4 °C for 15 min.
iii. Separation of the upper clear aqueous phase was followed by transfer into a new tube, where step II was repeated.
iv. The clear aqueous phase was transferred to a new tube, and an equal volume of isopropanol was added to the mixture, which was then briefly inverted for 5 seconds and incubated at room temperature for 10 min before being centrifuged for 10 min.
v. The supernatant was removed from the tube and 700 µl of 70% ethanol was added before centrifuging for 5 min (step was repeated three times
vi. After the supernatant was removed from the tube, the pellet was air-dried for 5-

10 min and placed on a heating block at 65°C for an additional 2-5 min. It was then re-suspended with 50 µl of an elution buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) (Qiagen-The Scientific Group).

### 3.2.5. Post-treatment of nucleic acid

Extracted nucleic acid was divided into three parts, of which the first group consisted of the standard PCR template, the second template was pre-heated at 55°C for 2 min and the last template was heated post-M-RTPCR assay.

## 3.3. Results

### 3.3.1. Screening of IAV (M) gene using rRT-PCR results

A total of 2144 faecal swab samples were collected for this study between January and February 2021 and then screened for the AIV M gene using the rRT-PCR assay. A total of 51 % (1083) of the samples tested positive for AIV RNA, while 49 % (1061) tested negative for virus particles. (See Figure 3.1.) Positive samples with a Ct value ≤ 31 were 30%, while those with a higher Ct ≥ 32 were 70%.

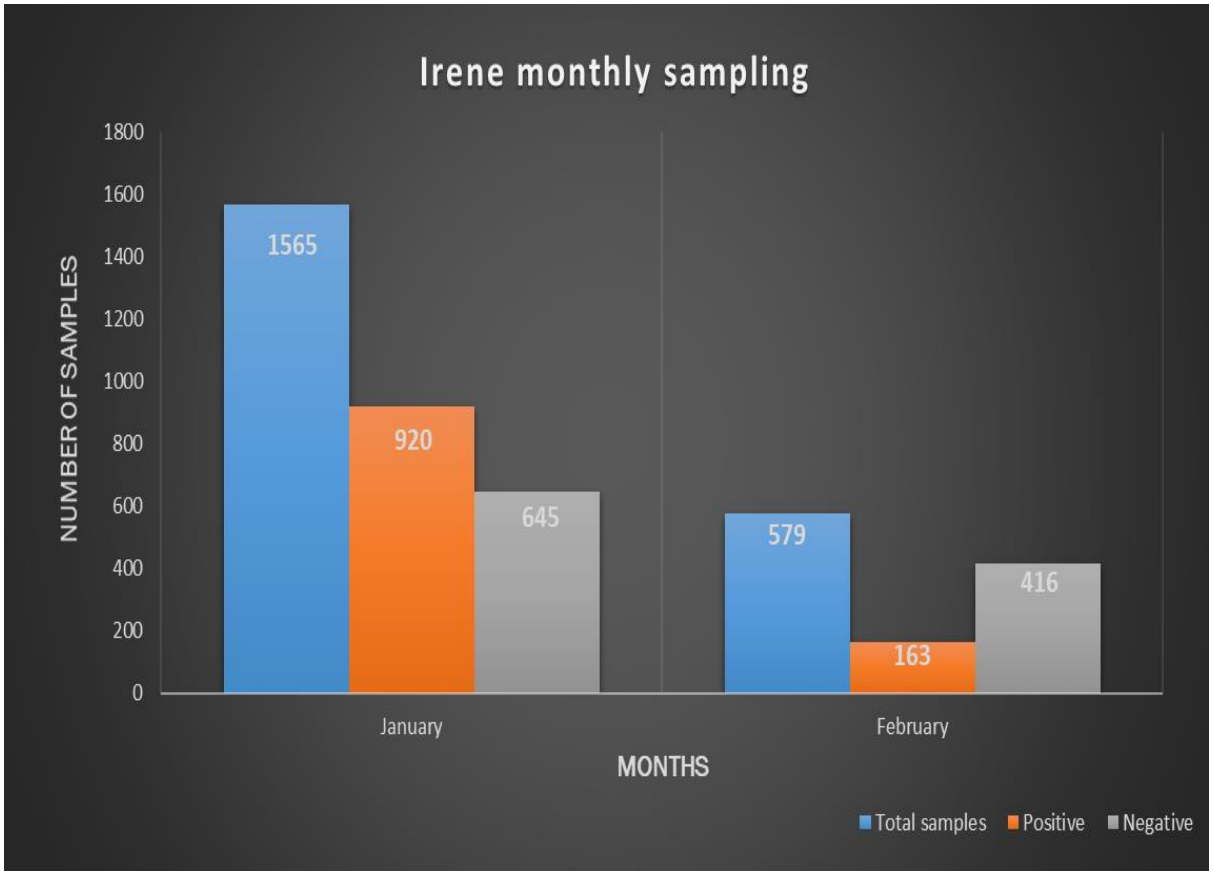


Figure 3.1: Total umbersof aecal swab samples collected and screened for IAV using rRT-PCR from January to February 2021.The blue block represents the total number of samples collected, the orange block represents samples that tested positive and the grey block represents samples that tested negative.

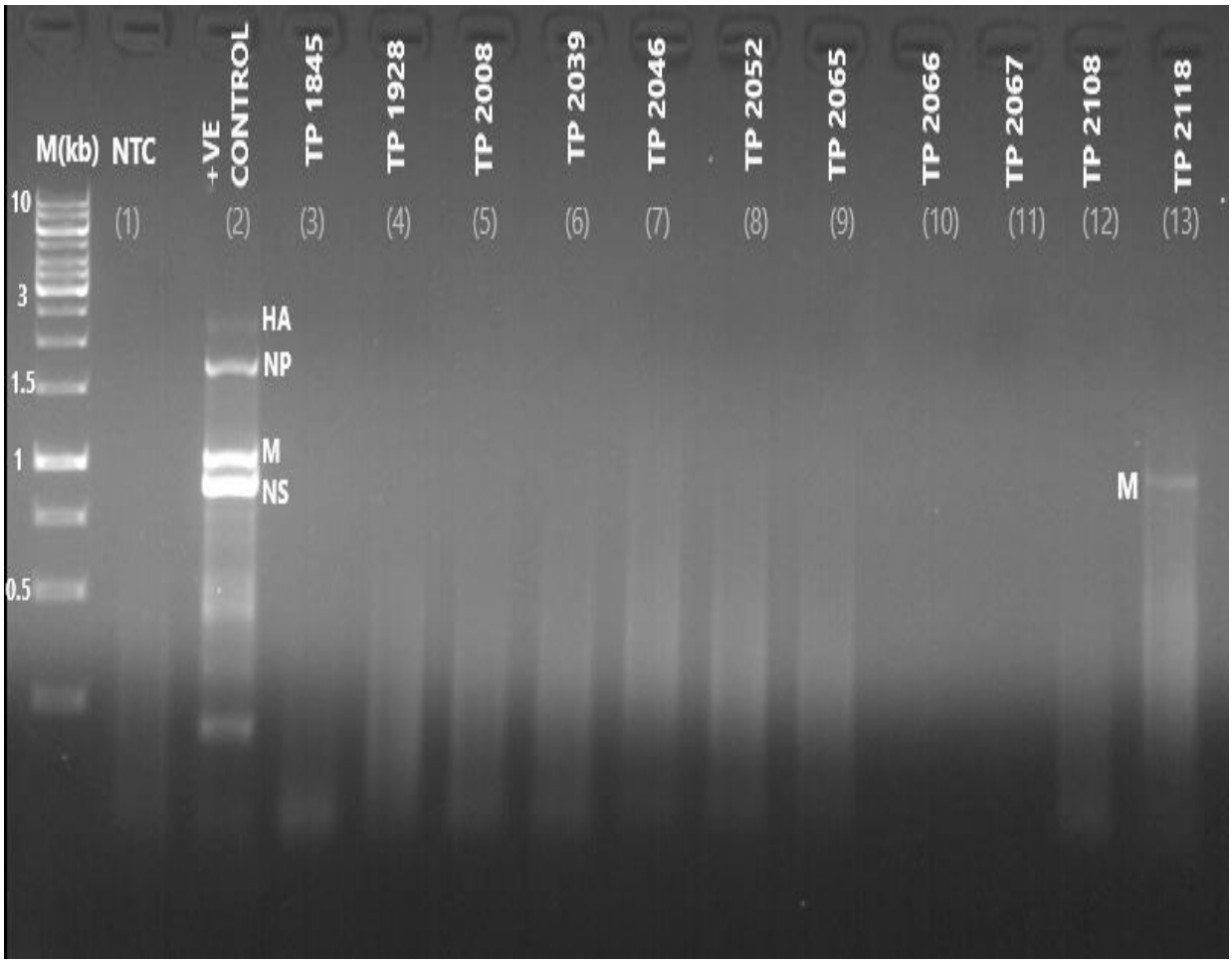


Figure 3.2: M-RTPCR on IAV positive faecal samples

Figure 3.2 shows the positive faecal swab samples by M-RTPCR. The Ct value of the samples are as follows according to the lane number: lane 3: TP 1845 (31.89); lane 4: TP 1928 (33.78); lane 5: TP 2008 (31.23); lane 6 TP 2039 (32.87); lane 7: TP 2046 (33.71); lane 8: TP 2052 (32.71); lane 9: TP 2065 (32.71); lane 10: TP 2066 (33.41); lane: 11 TP 2067 (24.19); lane 12: TP 2108 (32.55) and lane 13: TP 2118 (30.62). Lanes 3 to 12 showed no genome amplification, only faded smears were visible, and lanes 10 and 11 had no signs of amplification or smears present. Lane 13, which was TP 2118 shows a faint amplification of a genome segment although there was smear in the background.

### 3.3.2: Optimization and comparison of nucleic acid extraction of spiked faecal swabs

Table 3.1: Optimization of viral nucleic acid extraction adding RNA later ™ determined by rRT-PCR Ct value

| Dilution Factor | Trizol extraction method pre-M-RTPCR | Trizol extraction post M-RTPCR | IndiMag 48s pre-M-RTPCR | IndiMag 48s post-M-RTPCR |
|---|---|---|---|---|
| 1:2 | Undetermined | Undetermined | 19.54 | 21.36 |
| 1:4 | 26.52 | 29.25 | 20.33 | 22.45 |
| 1:8 | 26.9 | 25.39 | Undetermined | Undetermined |
| 1:16 | 27.39 | 33.57 | 21.93 | 23.92 |
| 1:32 | 27.57 | 32.2 | 23.63 | 25.99 |
| 1:64 | 27.33 | 31.58 | 25.8 | 28.77 |
| 1:128 | 27.3 | 33.43 | 26.48 | 29 |
| 1:256 | 26.76 | 33 | 27.48 | 31.23 |
| 1:512 | 28.24 | Undetermined | 27.78 | 32.07 |
| 1:1024 | 28.15 | 33.21 | 29.35 | 27.56 |
| Positive | 20.51 | 19.12 | 26.46 | 22.16 |

Table 3.1 shows the nucleic results of the IAV rRT-PCR analysis. The addition of RNA later™ increased viral nucleic acid recovery because both extraction methods had Ct values ≤ 30 before performing M-RTPCR. However, no results were obtained for serial dilution 1:2 before and after the M-RTPCR assay using manual extraction, and no results were obtained using the IndiMag 48s automated extraction for serial dilution 1:8. Extractions performed automatically with IndiMag 48s produced consistent and low Ct values.

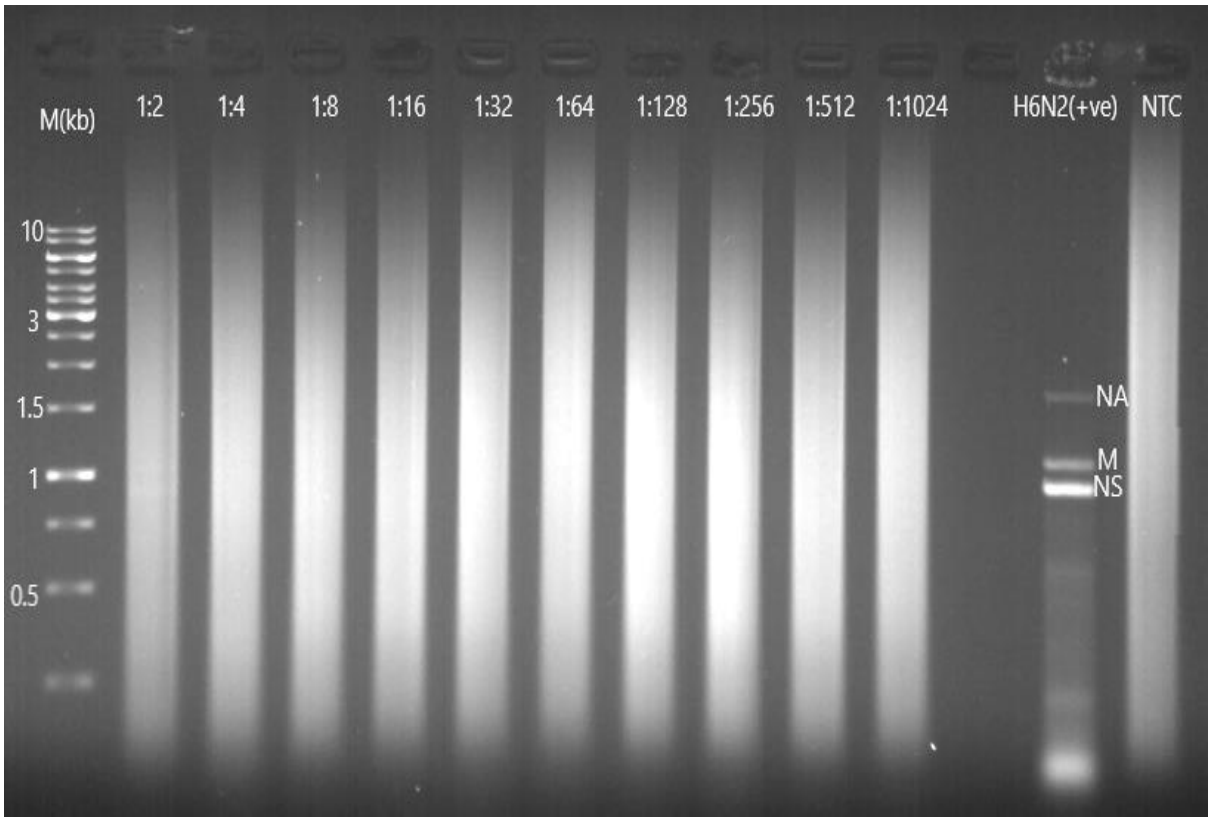


Figure 3.3: M-RTPCR results for Trizol extracted viral nucleic acid of the spiked faecal sample serial dilutions treated with RNA later™.

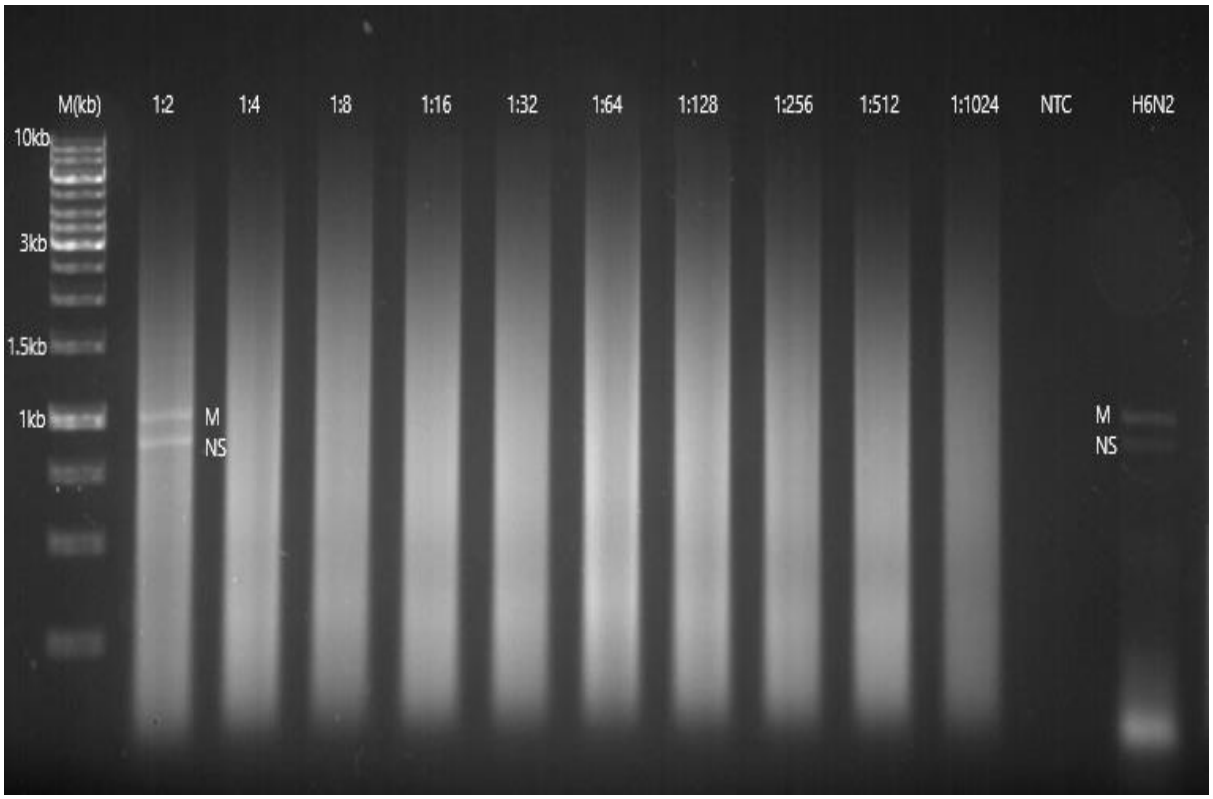


**Figure 3.4: M-RTPCR results for indiMag 48s automated extracted viral nucleic acid of a spiked faecal sample serial dilutions treated with RNA later.**

The M-RTPCR results for serial dilutions treated with RNA later™ are shown in Figures 3.3 and 3.4. Background inhibition due to faecal contamination was observed in both gels, resulting in smear formation and indicating the possibility of non-specific amplification, which could disguise IAV genome segments. Figure 3.4 shows faded genome segments and smears on the first lane which consists of 1:2 serial dilution gel despite the presence of contamination in the well.

### 3.3.3: Optimization of spiked faecal duck sample by pre-treating using filtration.

Table 3.2: Optimization of nucleic acid extraction from filtered serial dilution samples

| Dilution Factor | Trizol extraction method pre-M-RTPCR | Trizol extraction method value post M-RTPCR | IndiMag 48s pre-M-RTPCR | IndiMag 48s post M-RTPCR |
|---|---|---|---|---|
| 1:2 | 18.04 | 19.40 | 17.29 | 19.62 |
| 1:4 | undetermined | undetermined | 19.26 | 21.21 |
| 1:8 | 22.37 | 22.16 | 21.25 | 23.51 |
| 1:16 | 23.46 | 24.98 | 23.93 | 25.71 |
| 1:32 | 22.86 | 24.68 | 25.26 | 27.42 |
| 1:64 | 28.02 | undetermined | 26.26 | 27.45 |
| 1:128 | 26.24 | 27.93 | 26.86 | 28.12 |
| 1:256 | 25.23 | 27.94 | 27.58 | 28.10 |
| 1:512 | 27.5 | undetermined | 27.19 | 27.95 |
| 1:1024 | 22.75 | 28.09 | 27.45 | 29.64 |
| Positive | 19.10 | undetermined | 25.86 | 21.69 |

Based on the results obtained in Table 3.2 the pre-treatment method of filtering the samples proved to recover a high concentration of the viral nucleic acid. Both methods of extraction yielded Ct values ≤ 29 before the M-RTPCR assay, although the manual extraction method had more undetermined (negative) results in the dilution factor 1:4 before and after performing the M-RTPCR assay and in the dilution factors 1:64, 1:512 and the positive. Post M-RTPCR, the IndiMag 48s automated extraction recovered more IAV specific nucleic acid from the filtered faecal samples and the Ct values were consistently low and error-free.

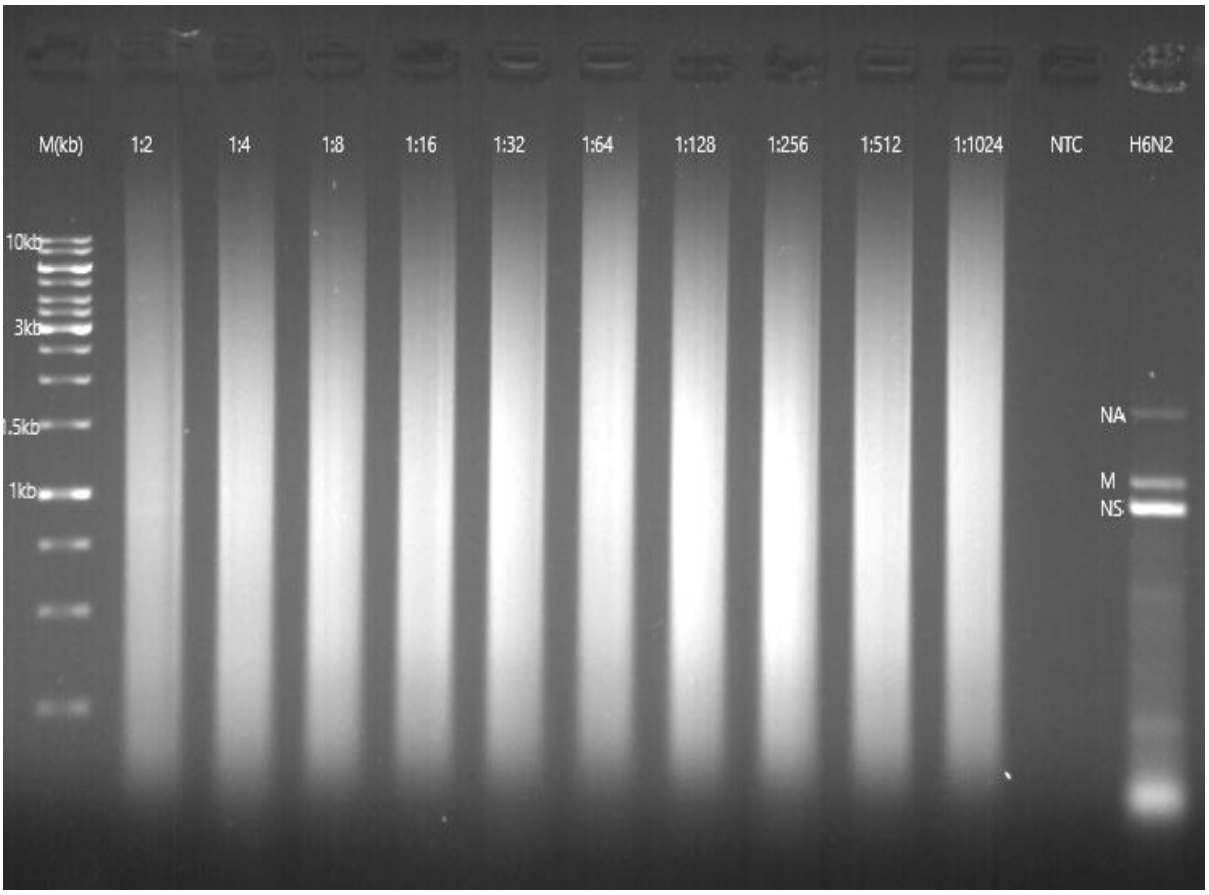


Figure 3.5: M-RTPCR results for filtered Trizol extracted nucleic acid from serial dilution of a spiked faecal duck sample

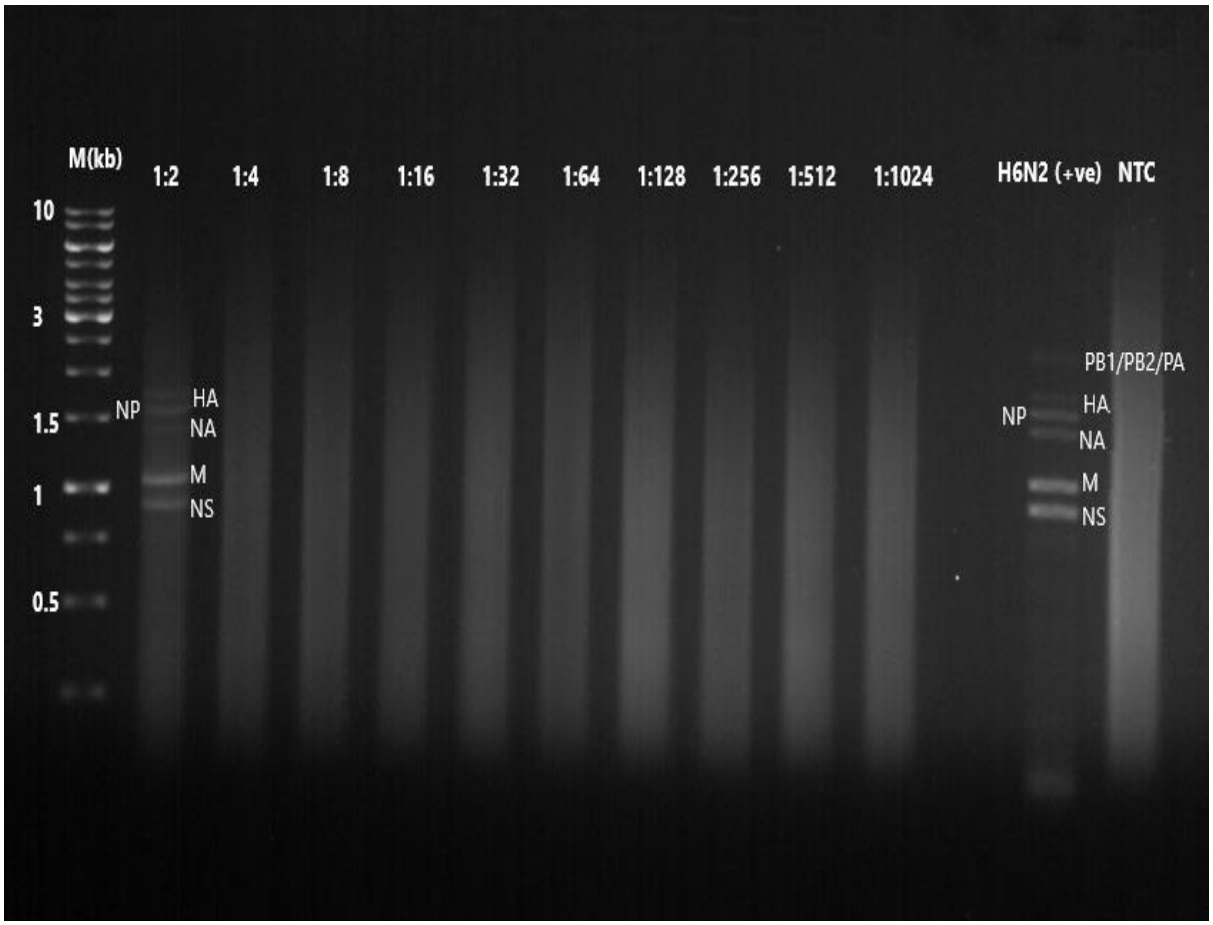


Figure 3.6: M-RTPCR results for filtered indiMag 48s extracted nucleic acid from serial dilution of a spiked faecal duck sample

A comparison of results obtained from manual and automated extraction of filtered treated faecal dilutions is presented in Figures 3.5 and 3.6 respectively. Both gels had smears in all the lanes, but only Figure 3.6 shows full genome amplification of the sample serially diluted 1:2 (lane 1). The gel in Figure 3.5 produced brighter smears with no indication of genome amplification except in the positive control.

### 3.3.4: Optimization of Trizol extraction method using Phenol-Chloroform Isoamyl in serially diluted spiked duck faecal samples

Table 3.3: Modified and standard Trizol extraction method and PCI

| Dilution Factor | Modified TRizol method | Modified TRizol post protocol |
|---|---|---|
| 1:2 | 11.9 | 19.35 |
| 1:4 | 24.43 | 24.60 |
| 1:8 | 25.99 | 30.06 |
| 1:16 | 27.78 | 30.38 |
| 1:32 | 29.51 | 30.69 |
| 1:64 | 27.32 | Undetermined |
| 1:128 | 30.36 | 34.03 |
| 1:256 | 30.23 | 34.72 |
| 1:512 | undetermined | Undetermined |
| 1:1024 | undetermined | Undetermined |
| Positive | 23.79 | 11.7 |

The results for serial dilutions of spiked faecal samples extracted using the modified Trizol method are shown in Table 3.3. The extraction method improved nucleic acid recovery and sensitivity, with Ct values of 31 obtained before the M-RTPCR assay. Both before and after the M-RTPCR, the serial dilutions 1:512 and 1:1024 were undetermined (negative), and the post-assay dilution factor 1:64 was also undetermined. Furthermore, after the M-RTPCR assay, the templates were amplified with serial dilutions ranging from 1:2 to 1:32, yielding Ct values less than 31, but 1:128 and 1:256 yielded lower values of less than 35.

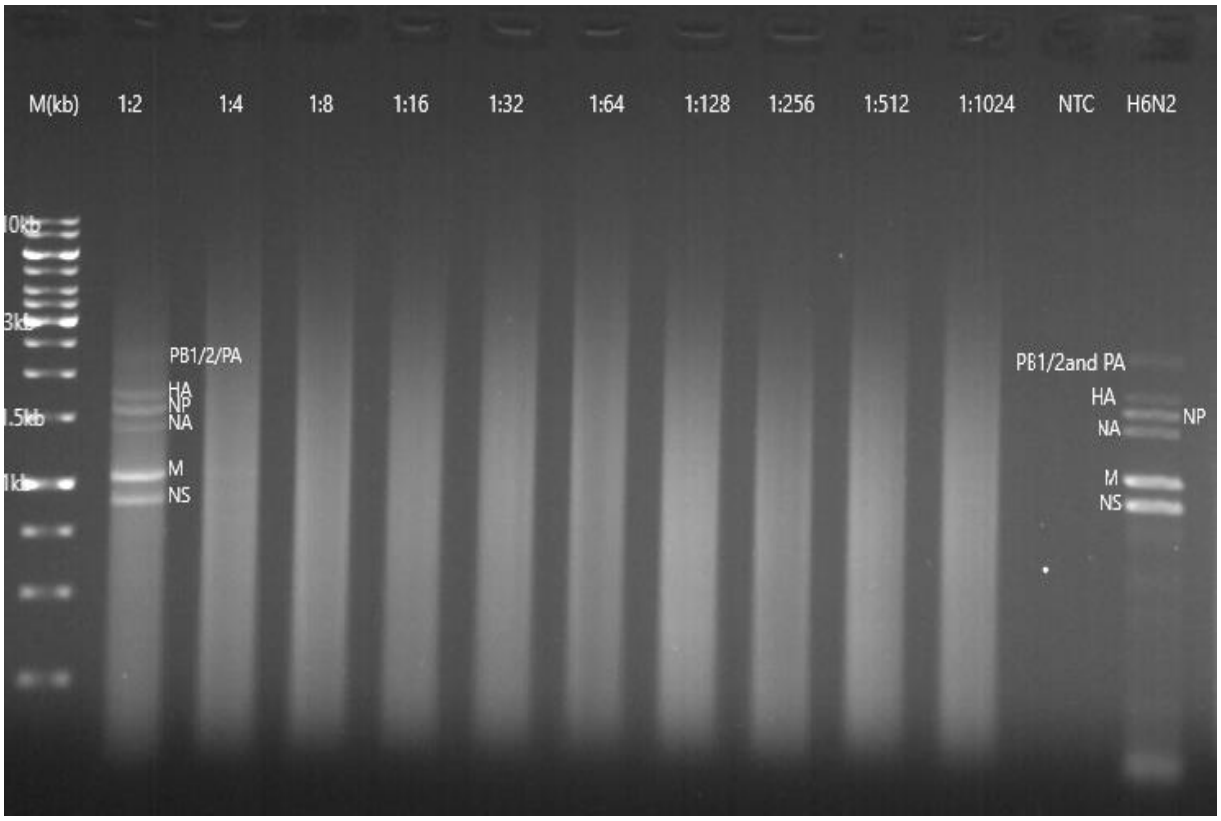


**Figure 3.7. M-RTPCR assay on serial dilutions of spiked faecal samples extracted using the modified Trizol extraction method.**

The results of the serial dilutions extracted with the modified Trizol method are shown in Figure 3.7. Faint smears were present on the gel from 1:4 to 1:1024 with no enhancement of genome amplification, howeve;, the 1:2 serial dilution demonstrated amplification of the full genome segments despite the presence of smears in the background. Furthermore, the 1:2 serial dilution had Ct value of 11.9, indicating a high recovery of viral nucleic acid from the sample; thus, after amplification, the sample still had a low Ct of 19.35, indicating a high presence of IAV viral RNA.

### 3.3.4. Post treatment of nucleic acid template

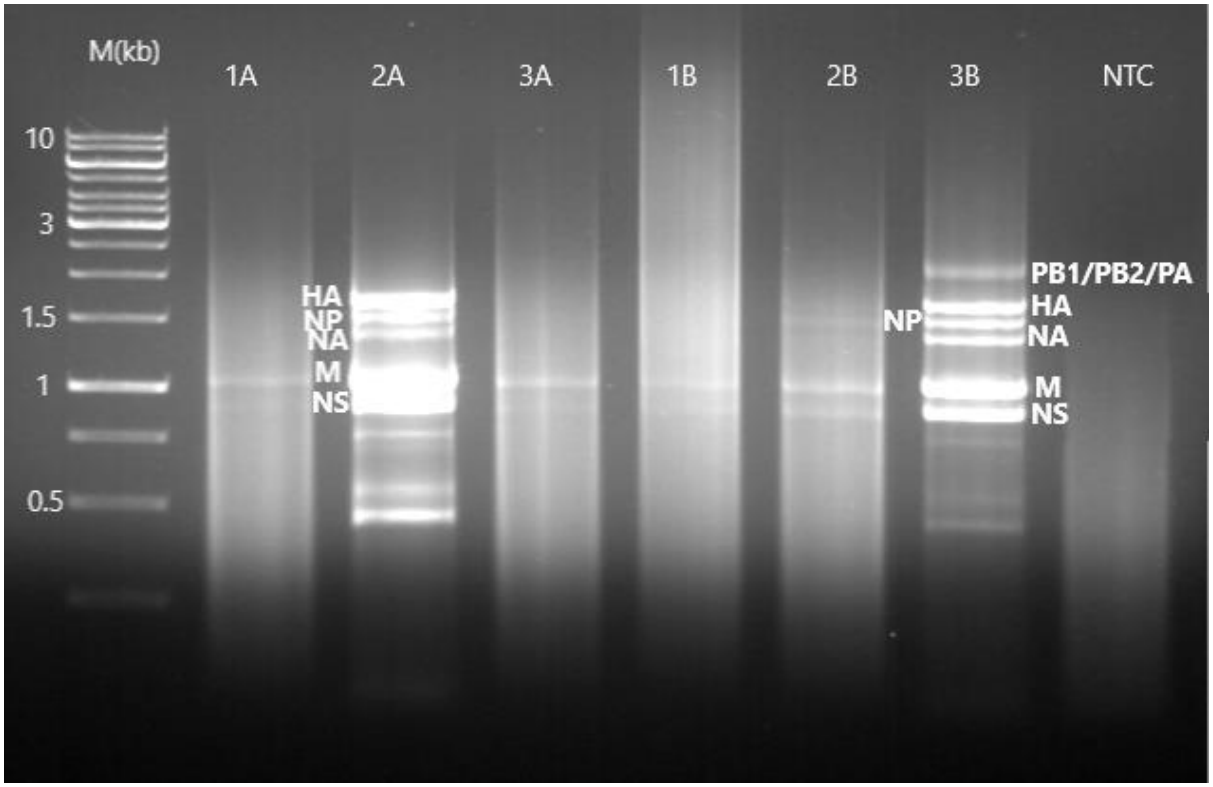


**Figure 3.8: M-RTPCR pre and post-heating results of nucleic acid extracted using modified Trizol method and IndiMag 48s. Nucleic acid labelled A were manually extracted while nucleic acid labelled B were extracted automatically.**

Figure 3.7 shows the results of the pre- and post-heating of the nucleic acid extracted from both the modified Trizol method and IndiMag 48s instrument. The first set of lanes are as follows: lane 1A: pre-heated samples, 2A: modified Trizol template and lane 3A is post-heating of the Trizol nucleic acid. While lane 1B is the pre-heated nucleic acid, 2B standard template and 3B consist of the post-heating of the nucleic acid. The nucleic acid extracted using both manual and automated instrument amplified genome segments in all wells, however, lane 3B contained full genome segments with the nucleic acid extracted using the automated instrument containing full genome segments of all eight IAV genes. In addition, the automated nucleic acid in well 1B and 2B amplified lower genome segments genes, which were still amplified and fairly visible when compared to the manually extracted nucleic acid.

Based on the overall results obtained from pre-treatment of the serial dilution of the spiked duck faecal sample, the addition of RNAlater™ and filtration of the samples yielded similar results with partial removal of contamination and inhibitors from samples. However, using the filtration process resulted in a higher yield of viral nucleic acid which was amplified for genome segments in both manual and automated extraction methods. In addition, when comparing the extraction process, the automated IndiMag 48s recovered higher viral nucleic acid from the samples with Ct values less ≤ 30 both before and after performing the M-RTPCR assay. Additionally, we show the amplification of genetic segments from the 1:2 serial dilution factor when the nucleic acid extracted from the automated process. Modified Trizol proved to be more reliable at recovering viral nucleic acids than standard Trizol since the wash step was repeated to remove any remaining inhibitors. This method demonstrated the ability to amplify full genome segments with a low Ct (≤20), thus possibly removing inhibitors.

Table 3.4: Comparison of positive samples extracted using indiMag 48s and modified Trizol method

| Sample ID | IndiMag 48s Sample Ct value | Modified Trizol Samples Ct value |
|---|---|---|
| TP 1845 | 31.89 | 34.37 |
| TP 2008 | 31.23 | Undetermined |
| TP 2067 | 24.19 | Undetermined |
| TP 2108 | 32.55 | 36.96 |
| TP 2118 | 24.83 | 30.62 |

The above table shows the comparison between the IndiMag 48 s and modified Trizol extracted viral nucleic acid. The viral RNA extracted from the IndiMAg 48s when used in the Rt-PCR had higher Ct values than those extracted manually. In addition, there were no irregularities with the nucleic acid extracted through the automated instrument. The samples with a Ct value less than 30 were subjected to M-RTPCR assay and heated post -reaction.

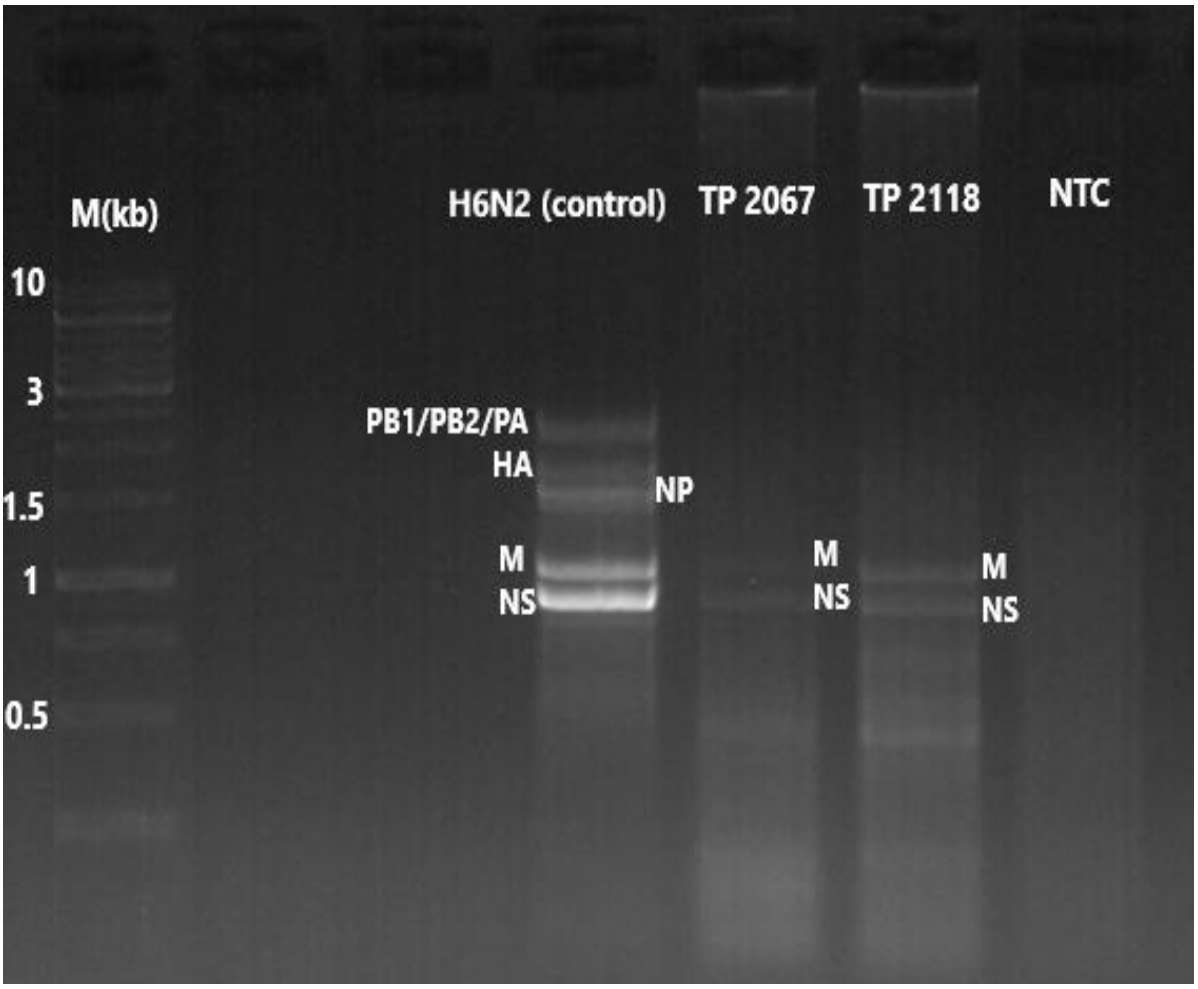


Figure 3.9: M-RTPCR results for positive faecal swab samples extracted using the IndiMag 48s.

Figure 3.8 shows the two positive faecal swab samples namely TP 2067 and TP 2118 which were tested on the MRT-PCR assay. Low molecular weight genome segments were obtained. Both samples had weak bands, TP 2067 had faded genome segments while TP 2118 produced faint slightly visible genome segments.

Table 3.5 Measuring of nucleic acid concentration using the Nanodrop instrument

| Sample ID | PCR template Ct | Nanodrop reading (ng /ul) | A260/280 |
|---|---|---|---|
| TP 2067 | 21.61 | 453.9 | 1.82 |
| TP 2118 | 14.61 | 444.4 | 1.84 |

After the amplification of TP 2067 and TP 2118, the M-RTPCR products were further tested using the RT-PCR to determine whether the genome enrichment had indeed occurred during the M-RTPCR reaction. The results obtained from the RT-PCR were presented in Table 3.5 and demonstrated that the PCR products generated a low Ct value below 22 indicating a higher viral load of the nucleic acid. Further analysis using the Nanodrop (Thermofischer) indicated that the nucleic acid measured had a 1.8 purity reading for both samples confirming the absence of inhibitors in the nucleic acid.

## 3.4. Discussion

A previous study indicated that a greater percentage of duck faecal samples tested positive for IAV than oropharyngeal swabs (Lickfett et al., 2018). In this part of the project, IAV was detected in 920 (85%) fresh faecal swabs in January, while 163 (28%) samples were positive in February .. Furthermore, 21 % (191), and as 1,8% (3) of the positive samples, had Ct values below 30 in January and February, respectively. Considering the results obtained, there was a significant drop in the number of positive samples obtained in February, which could have been caused by the ducks shedding less virus or the infection peak had passed. According to Kurmi et al.,(2013), the survivability of IAV depends on the environmental conditions and temperatures. Therefore, it could be possible that the exposure to wet weather could have resulted in the virus infected faecal samples acceleration in the inactivation process which then resulted in the negative effect by diluting the samples and washing away viral particles in the faeces.

Positive samples with a high viral load and generated low Ct values (≤25) were further subjected to M-RTPCR. Contamination and inhibitors that are naturally occurring in faecal samples, such as bile salts, host genomic DNA, bacteria and polysaccharides reduce the detectability and amplification of full genome segments (Das et al.,2009). To eliminate contamination in faecal swab samples, pre-treatment methods were compared. The first pre-treatment method involved adding RNA later™ to serially diluted samples, which were then briefly vortexed and incubated overnight at 4°C. Following the incubation period, the samples were divided into two groups, one of which was extracted with Trizol reagent using the standardized method, and the other was isolated using the IndiMag 48s automated instrument. The second pre-treatment method involved filtration of the samples using a 45 µm filter and syringe, with each sample filtered separately, before dividing the samples into two groups for the material and automated extraction process. The addition of RNA later™ and filtration were carried out separately, the results showed that the use of RNA later™ did recover a significant amount of viral RNA but was unsuccessful in removing inhibitors from the faecal swabs, even when using different extraction methods since smears were still present when the samples were assessed on agarose gel. The filtration recovered a higher yield of viral RNA from the serial dilutions with minimal contamination present, particularly the viral RNA extracted automatically. The manual method had more inconsistencies with the Ct values indicating that some of these samples had a higher presence of contamination which were not removed, thus preventing the virus from being detected. The automated extraction of viral RNA showed

a greater yield of the viral RNA was obtained, indicating that inhibitors or contamination were not present at a high concentration, and hence were able to be removed partially. Despite the smears that were produced on the filtration approach, the automated method was able to amplify full genome segments from samples with a low Ct value.

The results obtained from these methods showed that a significant amount of viral RNA was recovered, but were unsuccessful in removing the inhibitors because smears were constantly present, preventing the amplification of the full genome segments. Furthermore, the viral nucleic acid which was extracted using the automated instrument succeeded in amplifying genome segments despite the high presence of contamination and inhibitors in the background. The modified Trizol method was also successful in amplifying a full genome with at a very high Ct value, although when the method was applied in field faecal swab samples it displayed discrepancies and irregularities with the Ct, thus showing that the automated extraction method performs better than the manual extraction as it avoids cross-contamination that can arise due to handling of the samples throughout the extraction process. According to Das et al.,(2009) cloacal swabs and tissue samples contain organic materials and have complex chemical compositions, which makes it more difficult to extract RNA from.

From the initial positive IAV samples, only five (TP 1845; TP 2008; TP 2067;TP 2108; and TP 2118) had a Ct below 33 were re-extracted using the IndiMag 48s and modified Trizol method, I then compared the recovered viral RNA using the rRT-PCR assay. The viral RNA recovered using automated extraction was consistent with no discrepancies and had Ct ranging from 24.19 to 31.89, unlike the modified Trizol method. The viral RNA (namely TP 2067 and TP 2118) had lower Ct values were further subjected to M-RTPCR assay, of which the results showed both samples were successful in obtaining amplification of genome segments despite the faint smears that were still present in the background. The quality of the DNA from the M-RTPCR template was evaluated by measuring the absorbance ratio A260/A280, of which the DNA was considered to be sufficiently pure when the absorbance was 1.8. The DNA obtained from TP 2067 and TP 2118 were indicated to be adequate for sequencing and to determine the IAV subtypes.

# CHAPTER 4: IDENTIFICATION OF POSITIVE INFLUENZA A VIRUS SAMPLES USING NEXT GENERATION SEQUENCING

## 4.1. Introduction

Genome sequencing is a process that determines the order of nucleotides (A, U, C or G) in a single DNA sequence. Full-length genome sequencing can present comprehensive information for virus discovery, characterization, and genotyping (Lee, 2020). Previously, genome sequencing was for small RNA viruses, including influenza A, accomplished by "traditional" chain termination sequencing techniques such as Sanger sequencing (Lee, 2020).

Subsequent developments in next-generation sequencing (NGS) have transformed and improved the facilitation of complete viral genome sequencing from heterogeneous genetic material (Lee, 2020). NGS is a faster method for determining sequencing IAV genome than conventional sequencing since multiple fragments of nucleic acids can be analysed simultaneously.

Influenza A virus has a full-length genome that compromises approximately 13500 ribonucleotide sequences, with eight negative polarity segments (Lee, 2020). Because of the low fidelity of RNA polymerase and reassortment among different strains of influenza A, the genome is to have significant variability (Barzon et al., 2011, Bidzhieva et al., 2014, Van den Hoecke et al., 2015, Lahens et al., 2017, Huang et al., 2019). The genome nucleotides are sequenced at high coverage; however, a segmented genome prompts a technical challenge in obtaining full genome coverage (Van den Hoecke et al., 2015).

NGS methods are more sensitive, and they can identify the full diversity of viruses, including unidentified viruses (Barzon et al., 2011). The most frequently used NGS techniques are Illumina and Ion torrent platforms. Illumina sequencing uses a fluorescence-based paradigm for reading bases in a nucleotide sequence, this technique depends on the cyclic reversible termination which is less sensitive to homopolymer errors. The Illumina platform provides data that is suitable for *de novo* assembly and has long reads which allow deep sequencing, thus generating data sequences of the same length in a single run. However, this technique results in the reduction of the AT and GC rich regions, which causes an increase in sequencing errors (Lahens et al., 2017). The

Ion Torrent method employs pH measurements to read sequence nucleotide. It detects the hydrogen ions that are discharged as the dNTPs which combine and use a pH change sensor to detect a change in pH. This platform depends on a single nucleotide addition instead of using enzymatic approaches. Data sequence reads generated from Ion Torrent vary in size and length, in addition, the Ion Torrent platform has improved numerous types of chips and tools to change the performance for its investigative prerequisites (Lahens et al., 2017, Huang et al., 2019). These techniques continuously release updates as improved sequencing chemistry, nucleotide detection and high sensitivity throughput (Lahens et al., 2017). These technologies are frequently being used in many research studies because of the accuracy of the data generated by these platforms.

For this study, Ion torrent technology was applied for influenza A full genome sequencing. Positive field samples TP2118 and TP2067 obtained from Chapter 3 were subjected to full genome sequencing with sequence analysis to determine influenza A viral subtype.

## 4.2. Materials and Methods

### 4.2.1. Construction of an IAV reference sequence database

The reference gene segments (Table 1) for each of the 16xH and 9xN subtypes along with six internal protein-encoding gene segments were retrieved from the National Center for Biotechnology (NCBI) nucleotide database (https://www.ncbi.nhi.gov/nucleotide). The downloaded FASTA files were imported into the CLC workbench software v7.5.1, using the "import selection" and "standard import" options to a designated file.

### 4.2.2. Importation of NGS data to the CLC workbench software

The .bam files were downloaded from the FTP server at Stellenbosch University Central Analytical Facility thereafter imported into the CLC Genomics workbench software v7.5.1. The .bam read files were imported to the CLC Genomics workbench software using the "import" option and selecting the "standard import" subsequently saving the read files in the software.

### 4.2.3. Mapping of .bam reads against reference sequences

The. bam read file labelled TP2118 or TP2067 was highlighted, and the "toolbox" option was launched of which the "NGS core tools" was selected. The next selection was "map reads to references", of which all 32 reference sequences in Table 1 were selected. In the next selection panel, the option to retain default settings was chosen, but in the following panel was changed under the sub-heading "output options" thereby selecting "create stand-alone read mapping" in addition to "create report" options followed by "select and save files. The consensus sequence for each genome segment obtained for TP2118 and TP2067 was exported in FASTA format to a new folder.

Table 4.1: Reference sequences used for mapping read of samples sequences

| Gene segmentnumber | Gene/protein([1]abbr) | Subtype | Accession number |
|---|---|---|---|
| 1 | PB2 | | KY621531 |
| 2 | PB1, PB1-F2 | | KY621532 |
| 3 | PA, PA-X | | KY621533 |
| 4 | HA | H1 | MH637353 |
| | | H2 | MH637361 |
| | | H3 | KM054845 |
| | | H4 | MH637350 |
| | | H5 | KY621534 |
| | | H6 | GU122032 |
| | | H7 | KT777901 |
| | | H8 | MH412114 |
| | | H9 | KF313565 |
| | | H10 | KP287772 |
| | | H11 | MH637340 |
| | | H12 | KX101133 |
| | | H13 | HE802715 |
| | | H14 | JN696316 |
| | | H15 | KP087869 |
| | | H16 | HE802739 |
| 5 | NP | | KY621535 |
| 6 | NA | N1 | KJ484622 |
| | | N2 | KM244048 |
| | | N3 | MH411954 |
| | | N4 | CY080155 |
| | | N5 | KM244094 |
| | | N6 | MH637406 |
| | | N7 | KM244102 |
| | | N8 | KM244070 |
| | | N9 | MH637420 |
| 7 | M1 +M2 | | KY621537 |
| 8 | [2]NS allele_ A | | DQ376795 |
| | [3]NS allele_ B | | CY005780 |

[1]Abbr -Abbreviation of the genome segment. There are two genetic alleles of the NS protein: [2]allele A– represents avian and mammalian origin viruses; [3]allele B– represents only the avian viruses.

### 4.2.4. Verification of sequence identities through BLAST analysis

The FASTA files for each genome segment obtained for TP2118 and TP2067 were copied and pasted into the search box of the nucleotide sequence BLAST function. The settings on the BLAST nucleotide page were all left on default. The highest score with a matching description to the target strain was captured and tabulated for each genome segment.

## 4.3. Results

### 4.3.1. Assemble to reference results for TP2118

The Ion Torrent. bam read results for TP2118 generated a high-quality data analysis with a total of 425,317,942 bases, a Q score filter ≥ Q of 342,879,380, total reads of approximately 4,255,729 and lastly a mean read length of 99 bp.

The .bam reads were analysed by mapping read sequences against the 32 reference genome sequences in Table 4.1. The results generated for each .bam reads (TP2118) consisted of a consensus sequence for each genome segment.

Table 4.2: Assemble to reference results for TP2118

| Reference gene sequences number | Genome segment | Consensus length (nt) | Total read count | Average coverage | Percentage of Consensus sequence length | Reference length (nt) |
|---|---|---|---|---|---|---|
| **KY621531.1** | **PB2** | **1250** | **1722** | **133,60** | **54** | **2308** |
| **KY621532.1** | **PB1-F2** | **786** | **182** | **14,64** | **34** | **2309** |
| **KY621533.1** | **PA-X** | **2117** | **643** | **51,72** | **96** | **2200** |
| MH637353.1 | H1 | 66 | 3 | 0,06 | 3.7 | 1767 |
| MH637361.2 | H2 | 40 | 1 | 0,02 | 2 | 1689 |
| KM054845.1 | H3 | 0 | 0 | 0,00 | 0 | 1576 |
| MH637350.1 | H4 | 0 | 0 | 0,00 | 0 | 1713 |
| KY621534.1 | H5 | 0 | 0 | 0,00 | 0 | 1742 |
| GU122032.1 | H6 | 0 | 0 | 0,00 | 0 | 1635 |
| KT777901.1 | H7 | 47 | 12 | 0,23 | 2.8 | 1684 |
| MH412114.1 | H8 | 206 | 28 | 0,75 | 11.9 | 1735 |
| **KF313565.1** | **H9** | **1737** | **159** | **18,45** | **103** | **1683** |
| KP287772.1 | H10 | 64 | 10 | 0,23 | 3.8 | 1686 |
| MH637340 | H11 | 114 | 31 | 0,62 | 6.5 | 1748 |
| KX101133.1 | H12 | 60 | 1 | 0,03 | 3.5 | 1695 |
| HE802715.1 | H13 | 0 | 0 | 0,00 | 0 | 1736 |
| JN696316.2 | H14 | 111 | 289 | 4,42 | 6 | 1748 |
| KP087869.1 | H15 | 58 | 11 | 0,26 | 3 | 1713 |
| HE802739.1 | H16 | 183 | 53 | 1,14 | 10 | 1776 |

| KY621535.1 | NP | 1426 | 31 | 3,73 | 93 | 1529 |
|---|---|---|---|---|---|---|
| KJ484622.1 | N1 | 0 | 0 | 0,00 | 0 | 1350 |
| KM244048.1 | N2 | 969 | 147 | 18,28 | 67 | 1410 |
| MH411954.1 | N3 | 0 | 0 | 0,00 | 0 | 1428 |
| CY080155.1 | N4 | 0 | 0 | 0,00 | 0 | 1413 |
| KM244094.1 | N5 | 0 | 0 | 0,00 | 0 | 1422 |
| MH637406.1 | N6 | 0 | 0 | 0,00 | 0 | 1439 |
| KM244102.1 | N7 | 0 | 0 | 0,00 | 0 | 1413 |
| KM244070.1 | N8 | 0 | 0 | 0,00 | 0 | 1413 |
| MH637420.1 | N9 | 25 | 1 | 0,02 | 1.8 | 1374 |
| KY621537.1 | M1+M2 | 991 | 97 | 17,45 | 100 | 991 |
| DQ376795.1 | NS2 allele A | 900 | 1160 | 245,17 | 101 | 890 |
| CY005780.1 | NS2 allele B | 40 | 79 | 2,23 | 4 | 890 |

Sequence reads for TP2118 were mapped against each of the 16 HA types and 9 NA types to determine the subtype. The results showed eleven of the HA generated a consensus sequence, however, six of the HA types (H1; H2; H7; H10; H12; and H15) obtained low consensus length with low total read count and less coverage. Amongst the five remaining HA types, four generated partial sequences with low coverage and similarity percentage to the genome reference lengths (H8; H11; H14 and H16). The identified HA with an approximate similarity to the genome reference length was the H9, which generated a full genome of 1737 nt and a similarity percentage of 103 % to the reference length. In addition, the H9 had a high total read count of 159 and generated a high depth coverage of 18,45 amongst all generated HA types. Two of the NA types generated a consensus sequence however, only one was identified to have obtained a partial sequence consensus length, total read count and depth average coverage, whilst the other NA obtained low consensus length when compared to the reference length. The N2 has been identified as the NA type with a partial sequence of 969 nt and a similarity of 67 % when compared to the reference length, in addition, it generated a total read count of 147 and a depth average coverage of 18.28. Further analysis was conducted for the internal genome segments of which four consisted of partial sequences and two consisted of the full genome sequence. Firstly, PB2 generated a partial consensus sequence length of 1250 nt with a similarity of 54 % when compared against the reference length and obtained a total read count of 1722 with depth average coverage of

133.60. PB1-F2 which is the second internal gene segment generated a consensus length of 786 nt which contained a partial gene sequence of the reference length with a similarity of 34 %, and a total read count of 182 with depth coverage of 14,64. The PA-X gene segment generated a consensus length of 2117 nt which contained a partial sequence of the reference length with a high similarity of 96 %, and a total read count of 643 in addition to a depth average coverage of 51.72. The NP genome segment generated a partial consensus sequence of 9696 nt with high similarity of 93 % to the reference length and a total read count of 31 with a depth average coverage of 3.73. The remaining two genome segments M and NS both generated a full genome consensus sequence length of 991 nt and 900 nt respectively. Each genome segment obtained a similarity of 100% and 101%, with a total read count of 97 and 1160. Furthermore, a depth average coverage of 17,45 and 245,17 respectively.

### 4.3.2. Verification of sequence identity using BLAST analyses for TP2118

Table 4.3: BLAST analyses result for TP2118

| Description | Max Score | Total score | Query cover | Identity percentage | Accession length (nt) | Accessions number |
|---|---|---|---|---|---|---|
| Influenza A virus (A/pintail/Egypt/MBD-384C/2015(H3N6)) segment 1 PB2(PB2)<br><br>Query length: 1250 | 846 | 1823 | 95% | 94.69% | 2341 | MN208007 |
| Influenza A virus<br><br>(A/ duck /Mongolia/667/ 2019(H3N8)) segment 9 PB1 (PB1) and PB1-F2 protein (PB1-F2)<br><br>Query length: 786 | 652 | 1295 | 97% | 98.13% | 2326 | MT020228 |
| Influenza A virus<br><br>(A/chicken/Czech Republic/20617_2/2017<br><br>(H5N8) segment 3 polymerase (PA) and PA-X<br><br>Query Length :2200 | 4063 | 4063 | 100% | 100% | 2200 | KY621533 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Influenza A virus (A/duck/ Bangladesh/ 4493/2020(H9N 9) segment 4 (HA)<br><br>length :1737 | 2752 | 2752 | 96% | 96.36% | 1717 | MW749817 |
| Influenza A virus (A/duck/Moscow/ 5037/2014(H3N 8)) segment 5 (NP) Query length: 1426 | 1857 | 2216 | 100% | 93.76% | 1527 | MT773424 |
| Influenza A virus (A/ Muscovy duck/Pennsylvani a/4484-2/2013(N2)) segment 6 (NA) Query length:1410 | 2604 | 2604 | 100% | 100% | 1410 | KM244048 |
| Influenza A virus (A/chicken/Czech Republic/206-17_17_2/2017(H 5N8)) segment 7 (M2) gene and (M1)<br><br>Query Length:991 | 1831 | 1831 | 100% | 100% | 991 | HY621537 |
| Influenza A virus (A/mallard duck/ Netherlands/41/2 015(H5N1)) segment 8 (NS1)<br><br>Query Length: 900 | 1589 | 1589 | 98% | 98.88% | 890 | MF694125 |

In addition to analysing the generated data for each of the TP2118 genome segments described in Table 4.2, BLAST was used to identify the genetic origin for each gene. Six of the genome segments, including PB2, PB1-F2, H9, NP, N2, and NS1, were found to be from different waterfowl, while the PA-X and M genome segments were found to be from chicken. The genome segments come from all over the world, including Northeast Africa (PB2), East Asia (PB1-F2), South Asia (H9), and three from Europe (NP, N2, and M), with the chicken isolates coming from the Czech Republic (PA-X and M). Genome segments N2, M, and PA -X achieved 100% similarity compared to accession length, while PB1-F2, H9, and NS1 achieved greater than 95% similarity, and PB2 and NP had less than 95% similarity compared to accession length. Interestingly, genome segments identified in the Czech Republic were isolated from the H5N8 chicken subtype in 2017.

### 4.3.3. Assemble to reference results for TP2067

Ion Torrent. bam read results obtained for TP2067 generated a high-quality data analysis with a total of 495,056,995 bases, Q scores filter ≥ Q of 397,958,229, total read of approximately 4,717,432 and lastly mean read length of 104 bp.

The .bam reads were analysed by mapping read sequences against the 32 reference genome sequences in Table 4.4. The results generated for each .bam reads (TP2067) consisted of a consensus sequence for each genome segment.

Table 4.4: Assemble to reference results for TP2067

| Reference gene segment | Consensus length (nt) | Total read count | Average coverage | Percentage of Consensus sequence length | Reference Length (nt) |
|---|---|---|---|---|---|
| **KY621531.1 PB2** | **2046** | **4339** | **328,85** | **89** | **2308** |
| **KY621532.1 PB1-F2** | **2327** | **4360** | **321,75** | **101** | **2309** |
| **KY621533.1 PA_X** | **503** | **31** | **2,28** | **23** | **2200** |
| MH637353.1 H1 | 90 | 3 | 0,08 | 5 | 1767 |
| MH637361.2 H2 | 0 | 0 | 0,00 | 0 | 1689 |
| KM054845.1 H3 | 0 | 0 | 0,00 | 0 | 1576 |
| MH637350.1 H4 | 0 | 0 | 0,00 | 0 | 1713 |
| KY621534 H5 | 147 | 81 | 4,29 | 8 | 1742 |
| GU122032.1 H6 | 0 | 0 | 0,00 | | 1635 |
| KT777901.1 H7 | 38 | 4 | 0,007 | 2.3 | 1684 |
| MH412114.1 H8 | 129 | 22 | 0,64 | 7 | 1735 |
| KF313565.1 H9 | 0 | 0 | 0,00 | 0 | 1683 |
| KP287772.1 H10 | 52 | 1 | 0,03 | 3 | 1686 |
| MH637340 H11 | 65 | 23 | 0,48 | 3.7 | 1748 |
| KX101133.1 H12 | 0 | 0 | 0,00 | 0 | 1695 |
| HE802715.1 H13 | 77 | 2 | 0,04 | 4 | 1736 |
| JN696316.2 H14 | 48 | 114 | 1,67 | 2.7 | 1748 |
| KP087869.1 H15 | 62 | 12 | 0,28 | 3.6 | 1713 |
| HE802739.1 H16 | 181 | 39 | 0,76 | 10 | 1776 |
| **KY621535.1 NP** | **167** | **2** | **0,22** | **11** | **1529** |

| | | | | | |
|---|---|---|---|---|---|
| KJ484622.1 N1 | 194 | 15 | 1,72 | 14 | 1350 |
| KM244048.1 N2 | 0 | 0 | 0,00 | 0 | 1410 |
| MH411954.1 N3 | 0 | 0 | 0,00 | 0 | 1428 |
| CY080155.1 N4 | 0 | 0 | 0,00 | 0 | 1413 |
| KM244094.1 N5 | 0 | 0 | 0,00 | 0 | 1422 |
| MH637406.1 N6 | 0 | 0 | 0,00 | 0 | 1439 |
| KM244102.1 N7 | 29 | 1 | 0,02 | 2 | 1413 |
| KM244070.1 N8 | 0 | 0 | 0,00 | 0 | 1413 |
| MH637420.1 N9 | 0 | 0 | 0,00 | 0 | 1374 |
| **KY621537.1 M2_M1** | **560** | **127** | **22,10** | **57** | **991** |
| **DQ376795.1 NS2 allele A** | **900** | **545** | **107,52** | **101** | **890** |
| CY005780.1 NS<br>Allele B | 36 | 40 | 1,06 | 4 | 890 |

Sequence data for TP2067 were mapped against each of the 16 HA and 9 NA types to determine the subtypes. The results showed an overall of nine HA and two NA were obtained. Amongst the HA types generated seven produced a low consensus length with low total read count and depth average coverage. The three remaining HA types namely H5, H8 and H16 obtained average consensus sequence length with partial sequence to the reference length, and a similarity percentage of 8 %, 7 % and 10 %. Amongst these genome segments, the H5 generated a total read count of 81 and a depth average coverage of 4.29 which are slightly higher than H8 and H16. The NA types generated two consensus lengths of which the N1 obtained a partial sequence of 194 nt when compared to the reference length with a low total read count and depth coverage average of 0.22. However, due to the partial sequences and limited depths average coverage, obtained for HA and NA, we could not determine the full genome segment subtype. Further analysis was conducted for the internal genome segments, such as PB2 generated a consensus length of 2046 nt with partial sequences of the reference length and a similarity of 89%. In addition, the genome segment obtained a total read count of 4339 and depth coverage of 323,85. The genome segment PB1-F2 generated a consensus length of 2327 which included a full genome sequence with a total read of 4360 depths average coverage of 321,75 and similarity of 101 % to the reference length. Genome segment PA-X obtained a partial consensus sequence length of 503 with a similarity of 23 % to the reference length, a total read count of 31 and depth average coverage of 2.28. Further analysis was conducted for the NP genome segment which

generated a partial consensus sequence length of 167 nt with a low similarity of 11 % to the reference length, a total read count of 2 and depths coverage of 0.22. The M genome segment generated a partial consensus sequence length of 560 nt with a similarity of 57 % to the reference length, a total read count of 127 and depth average coverage of 22.10. Lastly, the NS genome segment generated a consensus length of approximately 900 nt with a similarity length of 101% to the reference length, furthermore, it obtained a total read count of 545 and depth average coverage of 107,52.

### 4.3.4. Verification of sequence identity using BLAST analyses for TP2067

Table.4.5: BLAST analyses result for TP2067

| Description | Max Score | Total score | Query cover | Identity percentage | Accession length (nt) | Accessions number |
|---|---|---|---|---|---|---|
| Influenza A virus (A/duck/Mongolia /30/2015(H3N8)) viral cRNA segment 1 (PB2) Query Length:2046 | 1253 | 2820 | 99% | 91.99% | 2341 | LC121233 |
| Influenza A virus (A/duck/Cambodia/10T-24-1-D14/2018(mixed)) segment PB1 and PB1-F2 protein. Query Length: 2321 | 3301 | 3666 | 100% | 92.44% | 2341 | MN703035 |
| Influenza A virus (A/duck/ Mongolia /820/2019(H4N2)) segment 3 PA protein PA-X genes. Query Length: 503 | 394 | 691 | 83% | 97.41% | 2216 | MT02053 |
| Influenza A virus (A/duck/Guangxi/ 112D4/2012(H3N2)) segment 5 NP gene. Query Length: 167 | 292 | 292 | 98% | 98.79% | 1565 | KT022271 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Influenza A virus (A/chicken/Kenya /A70-1403/2017(H9N 2)) segment 7 M2 gene. Query Length:581 | 612 | 973 | 92% | 98.83% | 991 | MN242738 |
| Influenza A virus (A/quail/Dubai/3 03/2000(H9N2)) segment 8 NS2 protein and NS1 gene. Query length: 900 | 1467 | 1467 | 98% | 96.4% | 895 | EF063540 |

BLAST analysis was performed on the data obtained for the identified genome segments in Table 4.4. For each genome segment, results were generated that included a detailed des cription of the sequence identity. Detailed analysis revealed that four of the genome seg ments (PB2; PB1-F2; PA-X; and NP) are from waterfowl, specifically duck, while the M genome segment is from chicken and the NS genome segment is from quail. These segments originated from different parts of the world, such as East Asia (PB2; PA -X and NP); Southeast Asia (PB1-F2); East Africa (M); and Western Asia (N2). In addition, two of the genome segments had less than 95% similarity and four had greater than 95% similarity compared to the accession length. Both genome segments M and NS were from the H9N2 subtype.

## 4.5. Discussion

The nucleotide sequence of influenza A strains was determined by sequencing, enabling precise identification and categorization of the various strains. NGS can be used to identify an identical strain of influenza A using a representative sequence, in addition to subtyping all influenza A viruses and detecting recurrent strains. By using NGS, it is possible to identify and obtain a complete sequence of the seasonal influenza A virus

Based on the results obtained from this chapter, TP2118 obtained full consensus sequences for HA (H9) and a partial consensus sequence for NA (N2) type. Furthermore, partial consensus sequences were obtained for four high molecular weight genome segments and two low molecular weight genome segments. For TP2067 there was no identification of the HA and NA types due to the partial consensus sequence obtained and limited total read and depth average coverage for the subtypes. TP2118 sample was identified to be H9N2 and named A/environment/South Africa/TP2118?2021 (H9N2).

H9N2 viruses are most commonly isolated from poultry but are also sporadically encountered by wild birds (Bergervoet et al., 2019, Suttie et al., 2019, Barberis et al., 2020). The H9 strain is referred to as a moderately pathogenic strain in chickens. There are three commonly detected NA strains in wild birds, namely N8 (26%), N6 (19%) and N3 (17%), while in poultry there are N2 (26 5), N7 (16%) and N4 (16%). The H8 and H9 with N4 and N2 are also the most frequently detected subtype pair of HA and NA (Bergervoet et al., 2019).

The H9N2 subtype is widespread throughout the world, especially in poultry and wild birds (Bhatta et al., 2020); nevertheless, since early 2000 it has been frequently detected in North Africa, where it has been discovered in Libya, Tunisia, Egypt and recently reported in Morocco (Awuni et al., 2019, Barberis et al., 2020). The first identification and isolation of H9N2 in South Africa occurred in farmed ostriches in the Cape region in 1995, although it was discovered that the virus was most likely a spillover from migrating birds (Abolnik et al., 2007). This subtype can affect poultry health and cause severe economic losses to the poultry industry, most commonly in cases of co-infection with other pathogens, irrespective of the pathogenic phenotype.

The spread of the H9N2 subtype in poultry has also caused documented infections in avian species and mammals such as pigs, horses, dogs, and humans. Consequently, this virus in different hosts in combination with another influenza A virus could provide several opportunities to become more virulent through the insidious spread, mutation and reassortment (Kaoud et al., 2014, Sun et al., 2020).

The H9N2 virus derived from TP2118 should be studied further because it is critical to track the strain's progress and gain a better understanding of ongoing zoonotic public health, which can help to reduce the danger of virus transmission to humans and other animals.

# CHAPTER 5: CONCLUSION

Surveillance for IAV is essential particularly in the study of wild waterfowl in wetland habitats since they are the primary reservoir. The surveillance of these birds is considered an early warning system to identify locations of higher risk and potentially discover new viral strains from the natural hosts. The waterfowl in the wetland study area congregate in high densities and often interact with various other bird species, thus resulting in the transmission of IAV strains. In addition, the wetland habitat has a high level of faecal samples that contain a high concentration of IAV. Screening of environmental faecal samples from a wetland study area is important because it can yield important data on IAV viral strains and infections in waterfowl and various other birds.

The study demonstrates that although faecal swab samples are ideal for the detection of IAV, these samples contain a high level of contaminants which could obstruct the full genome identification. Pre-treatment using filtration followed by the post-heating of the RNA template was successful in the partial removal of inhibitors and improved the amplification of the genome segments. In addition, the study showed that the modified M-RTPCR method is simple and cost-effective and can be widely used for faecal samples where virus isolation attempts have failed.

The identified positive faecal swab sample TP2118 was characterized as the H9N2 subtype, and the presence of this virus indicates the presence of a potentially zoonotic strain in a local community of waterfowl, in Gauteng Province. Surveillance at this study site should be continued using the methods optimized here.

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# APPENDICES

## Appendix A : rRT-PCR screening results for wild duck environmental samples

| Sample ID | Results | Ct Value |
|---|---|---|
| TP IRN_001 | Positive | 33.83 |
| TP IRN_002 | Negative | |
| TP IRN_003 | Positive | 30.56 |
| TP IRN_004 | Negative | |
| TP IRN_005 | Positive | 34.97 |
| TP IRN_006 | Positive | 35.85 |
| TP IRN_007 | Positive | 35.00 |
| TP IRN_008 | Positive | 36.21 |
| TP IRN_009 | Positive | 36.22 |
| TP IRN_010 | Positive | 26.81 |
| TP IRN_011 | Positive | 35.35 |
| TP IRN_012 | Positive | 34.72 |
| TP IRN_013 | Negative | |
| TP IRN_014 | Positive | 35.36 |
| TP IRN_015 | Negative | |
| TP IRN_016 | Positive | 31.54 |
| TP IRN_017 | Positive | 33.65 |
| TP IRN_018 | Negative | |
| TP IRN_019 | Negative | |
| TP IRN_020 | Positive | 36.52 |
| TP IRN_021 | Positive | 36.39 |
| TP IRN_022 | Positive | 34.14 |
| TP IRN_023 | Positive | 35.40 |
| TP IRN_024 | Positive | 35.20 |
| TP IRN_025 | Positive | 35.26 |
| TP IRN_026 | Negative | |
| TP IRN_027 | Negative | |
| TP IRN_028 | Positive | 32.70 |
| TP IRN_029 | Negative | |
| TP IRN_030 | Negative | |
| TP IRN_031 | Negative | |
| TP IRN_032 | Positive | 35.66 |
| TP IRN_033 | Positive | 36.37 |
| TP IRN_034 | Positive | 34.80 |
| TP IRN_035 | Negative | |
| TP IRN_036 | Positive | 34.10 |
| TP IRN_037 | Negative | |
| TP IRN_038 | Positive | 36.38 |
| TP IRN_039 | Positive | 35.37 |
| TP IRN_040 | Negative | |
| TP IRN_041 | Positive | 32.87 |
| TP IRN_042 | Positive | 34.59 |

| | | |
|---|---|---|
| TP IRN_043 | Positive | 35.91 |
| TP IRN_044 | Positive | 34.1 |
| TP IRN_045 | Negative | |
| TP IRN_046 | Positive | 30.80 |
| TP IRN_047 | Positive | 36.08 |
| TP IRN_048 | Negative | |
| TP IRN_049 | Negative | |
| TP IRN_050 | Positive | 34.47 |
| TP IRN_051 | Negative | |
| TP IRN_052 | Negative | |
| TP IRN_053 | Positive | 36.19 |
| TP IRN_054 | Negative | |
| TP IRN_055 | Negative | |
| TP IRN_056 | Positive | 35.30 |
| TP IRN_057 | Negative | |
| TP IRN_058 | Negative | |
| TP IRN_059 | Positive | 35.51 |
| TP IRN_060 | Negative | |
| TP IRN_061 | Positive | 34.64 |
| TP IRN_062 | Positive | 36.27 |
| TP IRN_063 | Negative | |
| TP IRN_064 | Positive | 34.50 |
| TP IRN_065 | Negative | |
| TP IRN_066 | Positive | 36.00 |
| TP IRN_067 | Negative | |
| TP IRN_068 | Negative | |
| TP IRN_069 | Positive | 34.96 |
| TP IRN_070 | Negative | |
| TP IRN_071 | Positive | 35.22 |
| TP IRN_072 | Positive | 36.15 |
| TP IRN_073 | Positive | 35.35 |
| TP IRN_074 | Positive | 36.35 |
| TP IRN_075 | Positive | 31.33 |
| TP IRN_076 | Negative | |
| TP IRN_077 | Negative | |
| TP IRN_078 | Negative | |
| TP IRN_079 | Negative | |
| TP IRN_080 | Negative | |
| TP IRN 081 | Negative | |
| TP IRN 082 | Negative | |
| TP IRN 083 | Negative | |
| TP IRN 084 | Negative | |
| TP IRN 085 | Negative | |
| TP IRN 086 | Negative | |
| TP IRN 087 | Negative | |
| TP IRN 088 | Negative | |
| TP IRN 089 | Positive | 33.27 |
| TP IRN 090 | Positive | 35.77 |
| TP IRN 091 | Positive | 34.71 |

| | | |
|---|---|---|
| TP IRN 092 | Negative | |
| TP IRN 093 | Negative | |
| TP IRN 094 | Positive | 31.11 |
| TP IRN 095 | Negative | |
| TP IRN 096 | Positive | 33.50 |
| TP IRN 097 | Positive | 31.53 |
| TP IRN 098 | Negative | |
| TP IRN 099 | Negative | |
| TP IRN 100 | Negative | |
| TP IRN 101 | Negative | |
| TP IRN 102 | Positive | 37.04 |
| TP IRN 103 | Positive | 33.63 |
| TP IRN 104 | Positive | 35.73 |
| TP IRN 105 | Positive | 28.98 |
| TP IRN 106 | Positive | 34.80 |
| TP IRN 107 | Positive | 32.09 |
| TP IRN 108 | Positive | 31.18 |
| TP IRN 109 | Positive | 33.99 |
| TP IRN 110 | Negative | |
| TP IRN 111 | Positive | 35.77 |
| TP IRN 112 | Positive | 34.50 |
| TP IRN 113 | Negative | |
| TP IRN 114 | Positive | 34.28 |
| TP IRN 115 | Negative | |
| TP IRN 116 | Negative | |
| TP IRN 117 | Positive | 35.93 |
| TP IRN 118 | Positive | 31.99 |
| TP IRN 119 | Negative | |
| TP IRN 120 | Positive | 36.72 |
| TP IRN 121 | Negative | |
| TP IRN 122 | Negative | |
| TP IRN 123 | Positive | 33.74 |
| TP IRN 124 | Positive | 27.55 |
| TP IRN 125 | Negative | |
| TP IRN 126 | Positive | 34.09 |
| TP IRN 127 | Positive | 35.36 |
| TP IRN 128 | Positive | 30.20 |
| TP IRN 129 | Negative | |
| TP IRN 130 | Negative | |
| TP IRN 131 | Negative | |
| TP IRN 132 | Negative | |
| TP IRN 133 | Positive | 31.44 |
| TP IRN 134 | Positive | 32.35 |
| TP IRN 135 | Positive | 35.81 |
| TP IRN 136 | Negative | |
| TP IRN 137 | Negative | |
| TP IRN 138 | Positive | 33.84 |
| TP IRN 139 | Positive | 35.35 |
| TP IRN 140 | Positive | 36.98 |

| | | |
|---|---|---|
| TP IRN 141 | Negative | |
| TP IRN 142 | Positive | 35.90 |
| TP IRN 143 | Positive | 35.77 |
| TP IRN 144 | Negative | |
| TP IRN_145 | Negative | |
| TP IRN_146 | Positive | 34.80 |
| TP IRN_147 | Negative | |
| TP IRN_148 | Negative | |
| TP IRN_149 | Positive | 36.27 |
| TP IRN_150 | Negative | |
| TP IRN_151 | Positive | 36.17 |
| TP IRN_152 | Negative | |
| TP IRN_153 | Positive | 36.55 |
| TP IRN_154 | Negative | |
| TP IRN_155 | Negative | |
| TP IRN_156 | Positive | 35.63 |
| TP IRN_157 | Positive | 36.62 |
| TP IRN_158 | Positive | 36.62 |
| TP IRN_159 | Positive | 35.62 |
| TP IRN_160 | Negative | |
| TP IRN_161 | Negative | |
| TP IRN_162 | Negative | |
| TP IRN_163 | Negative | |
| TP IRN_164 | Negative | |
| TP IRN_165 | Negative | |
| TP IRN_166 | Negative | |
| TP IRN_167 | Negative | |
| TP IRN_168 | Negative | |
| TP IRN_169 | Positive | 36.51 |
| TP IRN_170 | Negative | |
| TP IRN_171 | Negative | |
| TP IRN_172 | Negative | |
| TP IRN_173 | Positive | 36.37 |
| TP IRN_174 | Negative | |
| TP IRN_175 | Positive | 36.47 |
| TP IRN_176 | Negative | |
| TP IRN_177 | Positive | 35.39 |
| TP IRN_178 | Positive | 35.67 |
| TP IRN_179 | Negative | |
| TP IRN_180 | Negative | |
| TP IRN_181 | Negative | |
| TP IRN_182 | Positive | 36.47 |
| TP IRN_183 | Positive | 35.21 |
| TP IRN_184 | Negative | |
| TP IRN_185 | Negative | 36.38 |
| TP IRN_186 | Negative | |
| TP IRN_187 | Negative | |
| TP IRN_188 | Negative | |
| TP IRN_189 | Negative | |

| | | |
|---|---|---|
| TP IRN_190 | Negative | |
| TP IRN_191 | Negative | |
| TP IRN_192 | Negative | |
| TP IRN_193 | Negative | |
| TP IRN_194 | Negative | |
| TP IRN_195 | Positive | 36.65 |
| TP IRN_196 | Positive | 36.55 |
| TP IRN_197 | Positive | 36.55 |
| TP IRN_198 | Negative | |
| TP IRN_199 | Positive | 35.58 |
| TP IRN_200 | Negative | |
| TP IRN_201 | Positive | 36.77 |
| TP IRN_202 | Negative | |
| TP IRN_203 | Negative | |
| TP IRN_204 | Negative | |
| TP IRN_205 | Positive | 35.66 |
| TP IRN_206 | Positive | 35.75 |
| TP IRN_207 | Negative | |
| TP IRN_208 | Positive | 31.73 |
| TP IRN_209 | Positive | 35.40 |
| TP IRN_210 | Negative | |
| TP IRN_211 | Negative | |
| TP IRN_212 | Positive | 34.97 |
| TP IRN_213 | Positive | 35.46 |
| TP IRN_214 | Positive | 36.42 |
| TP IRN_215 | Negative | |
| TP IRN_216 | Negative | |
| TP IRN_217 | Negative | |
| TP IRN_218 | Negative | |
| TP IRN_219 | Positive | 33.80 |
| TP IRN_220 | Negative | |
| TP IRN_221 | Negative | |
| TP IRN_222 | Positive | 36.91 |
| TP IRN_223 | Positive | 36.34 |
| TP IRN_224 | Negative | |
| TP IRN_225 | Negative | |
| TP IRN_226 | Negative | |
| TP IRN_227 | Negative | |
| TP IRN_228 | Negative | |
| TP IRN_229 | Negative | |
| TP IRN_230 | Negative | |
| TP IRN_231 | Negative | |
| TP IRN_232 | Negative | |
| TP IRN_233 | Negative | |
| TP IRN_234 | Positive | 36.59 |
| TP IRN_235 | Positive | 35.64 |
| TP IRN_236 | Negative | |
| TP IRN_237 | Positive | 32.27 |
| TP IRN_238 | Negative | |

| | | |
|---|---|---|
| TP IRN_239 | Positive | 36.64 |
| TP IRN_240 | Positive | 33.22 |
| TP IRN_241 | Positive | 34.40 |
| TP IRN_242 | Positive | 31.38 |
| TP IRN_243 | Negative | |
| TP IRN_244 | Negative | |
| TP IRN_245 | Negative | |
| TP IRN_246 | Negative | |
| TP IRN_247 | Negative | |
| TP IRN_248 | Negative | |
| TP IRN_249 | Negative | |
| TP IRN_250 | Negative | |
| TP IRN_251 | Positive | 35.70 |
| TP IRN_252 | Positive | 36.96 |
| TP IRN_253 | Negative | |
| TP IRN_254 | Positive | 32.63 |
| TP IRN_255 | Negative | |
| TP IRN_256 | Negative | |
| TP IRN_257 | Negative | |
| TP IRN_258 | Positive | 36.39 |
| TP IRN_259 | Negative | |
| TP IRN_260 | Negative | |
| TP IRN_261 | Negative | |
| TP IRN_262 | Negative | |
| TP IRN_263 | Negative | |
| TP IRN_264 | Positive | 34.39 |
| TP IRN_265 | Negative | |
| TP IRN_266 | Negative | |
| TP IRN_267 | Positive | 33.95 |
| TP IRN_268 | Positive | 35.53 |
| TP IRN_269 | Positive | 36.57 |
| TP IRN_270 | Positive | 35.02 |
| TP IRN_271 | Positive | 36.69 |
| TP IRN_272 | Positive | 35.46 |
| TP IRN_273 | Positive | 31.78 |
| TP IRN_274 | Negative | |
| TP IRN_275 | Positive | 35.29 |
| TP IRN_276 | Positive | 35.22 |
| TP IRN_277 | Negative | |
| TP IRN_278 | Positive | 33.27 |
| TP IRN_279 | Positive | 36.50 |
| TP IRN_280 | Positive | 36.50 |
| TP IRN_281 | Negative | |
| TP IRN_282 | Negative | |
| TP IRN_283 | Positive | 34.35 |
| TP IRN_284 | Negative | |
| TP IRN_285 | Positive | 34.65 |
| TP IRN_286 | Negative | |
| TP IRN_287 | Negative | |

| | | |
|---|---|---|
| TP IRN_288 | Negative | |
| TP IRN_289 | Positive | 35.38 |
| TP IRN_290 | Negative | |
| TP IRN_291 | Positive | 34.49 |
| TP IRN_292 | Negative | |
| TP IRN_293 | Negative | |
| TP IRN_294 | Positive | 33.54 |
| TP IRN_295 | Negative | |
| TP IRN_296 | Positive | 35.49 |
| TP IRN_297 | Positive | 36.57 |
| TP IRN_298 | Positive | 35.49 |
| TP IRN_299 | Positive | 30.77 |
| TP IRN_300 | Positive | 36.41 |
| TP IRN_301 | Negative | |
| TP IRN_302 | Positive | 36.65 |
| TP IRN_303 | Positive | 35.63 |
| TP IRN_304 | Negative | |
| TP IRN_305 | Positive | 36.58 |
| TP IRN_306 | Negative | |
| TP IRN_307 | Negative | |
| TP IRN_308 | Negative | |
| TP IRN_309 | Positive | 36.02 |
| TP IRN_310 | Negative | |
| TP IRN_311 | Negative | |
| TP IRN_312 | Positive | 19.98 |
| TP IRN_313 | Positive | 31.44 |
| TP IRN_314 | Negative | |
| TP IRN_315 | Positive | 34.87 |
| TP IRN_316 | Negative | |
| TP IRN_317 | Negative | |
| TP IRN_318 | Negative | |
| TP IRN_319 | Positive | 36.32 |
| TP IRN_320 | Negative | |
| TP IRN_321 | Negative | |
| TP IRN_322 | Positive | 31.40 |
| TP IRN_323 | Negative | |
| TP IRN_324 | Negative | |
| TP IRN_325 | Positive | 35.39 |
| TP IRN_326 | Negative | |
| TP IRN_327 | Negative | |
| TP IRN_328 | Negative | |
| TP IRN_329 | Negative | |
| TP IRN_330 | Negative | |
| TP IRN_331 | Negative | |
| TP IRN_332 | Positive | 34.35 |
| TP IRN_333 | Positive | 31.29 |
| TP IRN_334 | Negative | |
| TP IRN_335 | Negative | |
| TP IRN_336 | Positive | 25.77 |

| | | |
|---|---|---|
| TP IRN_337 | Positive | 36.94 |
| TP IRN_338 | Positive | 36.45 |
| TP IRN_339 | Negative | |
| TP IRN_340 | Negative | |
| TP IRN_341 | Negative | |
| TP IRN_342 | Negative | |
| TP IRN_343 | Negative | |
| TP IRN_344 | Negative | |
| TP IRN_345 | Negative | |
| TP IRN_346 | Positive | 30.17 |
| TP IRN_347 | Negative | |
| TP IRN_348 | Positive | 29.73 |
| TP IRN_349 | Positive | 35.13 |
| TP IRN_350 | Negative | |
| TP IRN_351 | Negative | |
| TP IRN_352 | Positive | 32.27 |
| TP IRN_353 | Positive | 32.26 |
| TP IRN_354 | Negative | |
| TP IRN_355 | Positive | 35.39 |
| TP IRN_356 | Positive | 34.91 |
| TP IRN_357 | Positive | 36.89 |
| TP IRN_358 | Positive | 30.49 |
| TP IRN_359 | Negative | |
| TP IRN_360 | Negative | |
| TP IRN_361 | Negative | |
| TP IRN_362 | Negative | |
| TP IRN_363 | Negative | |
| TP IRN_364 | Negative | |
| TP IRN_365 | Positive | 34.23 |
| TP IRN_366 | Negative | |
| TP IRN_367 | Negative | |
| TP IRN_368 | Negative | |
| TP IRN_369 | Negative | |
| TP IRN_370 | Negative | |
| TP IRN_371 | Negative | |
| TP IRN_372 | Negative | |
| TP IRN_373 | Negative | |
| TP IRN_374 | Positive | 32.54 |
| TP IRN_375 | Negative | |
| TP IRN_376 | Negative | |
| TP IRN_377 | Negative | |
| TP IRN_378 | Negative | |
| TP IRN_379 | Negative | |
| TP IRN_380 | Negative | |
| TP IRN_381 | Negative | |
| TP IRN_382 | Negative | |
| TP IRN_383 | Negative | |
| TP IRN_384 | Negative | |
| TP IRN_385 | Negative | |

| | | |
|---|---|---|
| TP IRN_386 | Positive | 31.68 |
| TP IRN_387 | Negative | |
| TP IRN_388 | Negative | |
| TP IRN_389 | Negative | |
| TP IRN_390 | Negative | |
| TP IRN_391 | Negative | |
| TP IRN_392 | Negative | |
| TP IRN_393 | Negative | |
| TP IRN_394 | Negative | |
| TP IRN_395 | Negative | |
| TP IRN_396 | Positive | 32.39 |
| TP IRN_397 | Negative | |
| TP IRN_398 | Negative | |
| TP IRN_399 | Positive | 29.15 |
| TP IRN_400 | Positive | 37.39 |
| TP IRN_401 | Negative | |
| TP IRN_402 | Negative | |
| TP IRN_403 | Positive | 36.21 |
| TP IRN_404 | Positive | 32.93 |
| TP IRN_405 | Positive | 30.65 |
| TP IRN_406 | Negative | |
| TP IRN_407 | Positive | 28.14 |
| TP IRN_408 | Positive | 32.19 |
| TP IRN_409 | Negative | |
| TP IRN_410 | Negative | |
| TP IRN_411 | Positive | 32.98 |
| TP IRN_412 | Negative | |
| TP IRN_413 | Negative | |
| TP IRN_414 | Positive | 32.66 |
| TP IRN_415 | Positive | 33.52 |
| TP IRN_416 | Positive | 33.91 |
| TP IRN_417 | Positive | 31.23 |
| TP IRN_418 | Positive | 34.48 |
| TP IRN_419 | Negative | |
| TP IRN_420 | Negative | |
| TP IRN_421 | Positive | 28.27 |
| TP IRN_422 | Positive | 29.60 |
| TP IRN_423 | Positive | 34.90 |
| TP IRN_424 | Positive | 33.82 |
| TP IRN_425 | Positive | 31.22 |
| TP IRN_426 | Negative | |
| TP IRN_427 | Negative | |
| TP IRN_428 | Positive | 10.00 |
| TP IRN_429 | Positive | 36.44 |
| TP IRN_430 | Positive | |
| TP IRN_431 | Positive | 31.48 |
| TP IRN_432 | Positive | |
| TP IRN_433 | Positive | 29.84 |
| TP IRN_434 | Positive | 33.46 |

| | | |
|---|---|---|
| TP IRN_435 | Positive | 32.72 |
| TP IRN_436 | Positive | |
| TP IRN_437 | Positive | |
| TP IRN_438 | Positive | |
| TP IRN_439 | Positive | 27.72 |
| TP IRN_440 | Positive | 32.79 |
| TP IRN_441 | Positive | 30.75 |
| TP IRN_442 | Positive | |
| TP IRN_443 | Positive | |
| TP IRN_444 | Positive | 29.89 |
| TP IRN_445 | Positive | 34.27 |
| TP IRN_446 | Positive | |
| TP IRN_447 | Positive | 34.40 |
| TP IRN_448 | Positive | 30.44 |
| TP IRN_449 | Positive | 27.56 |
| TP IRN_450 | Positive | 33.55 |
| TP IRN_451 | Positive | 32.63 |
| TP IRN_452 | Positive | 32.88 |
| TP IRN_453 | Positive | 33.99 |
| TP IRN_454 | Positive | 33.19 |
| TP IRN_455 | Positive | 30.11 |
| TP IRN_456 | Positive | 34.02 |
| TP IRN_457 | Positive | 30.85 |
| TP IRN_458 | Positive | 33.52 |
| TP IRN_459 | Positive | 32.28 |
| TP IRN_460 | Positive | 34.31 |
| TP IRN_461 | Positive | 33.99 |
| TP IRN_462 | Negative | |
| TP IRN_463 | Positive | 33.65 |
| TP IRN_464 | Positive | 31.57 |
| TP IRN_465 | Positive | 31.36 |
| TP IRN_466 | Negative | |
| TP IRN_467 | Positive | 29.85 |
| TP IRN_468 | Positive | 32.29 |
| TP IRN_469 | Positive | 31.59 |
| TP IRN_470 | Positive | 29.88 |
| TP IRN_471 | Positive | 28.79 |
| TP IRN_472 | Positive | 31.82 |
| TP IRN_473 | Positive | 28.89 |
| TP IRN 474 | Negative | |
| TP IRN 475 | Negative | |
| TP IRN 476 | Negative | |
| TP IRN 477 | Positive | 31.99 |
| TP IRN 478 | Negative | |
| TP IRN 479 | Positive | 33.27 |
| TP IRN 480 | Positive | 33.82 |
| TP IRN 481 | Positive | 32.55 |
| TP IRN 482 | Negative | |
| TP IRN 483 | Negative | |

| | | |
|---|---|---|
| TP IRN 484 | Positive | 35.63 |
| TP IRN 485 | Positive | 36.52 |
| TP IRN 486 | Positive | 36.53 |
| TP IRN 487 | Positive | 32.19 |
| TP IRN 488 | Positive | 33.27 |
| TP IRN 489 | Positive | 32.73 |
| TP IRN 490 | Positive | 32.97 |
| TP IRN 491 | Positive | 35.68 |
| TP IRN 492 | Positive | 34.08 |
| TP IRN 493 | Negative | |
| TP IRN 494 | Positive | 36.90 |
| TP IRN 495 | Positive | 28.51 |
| TP IRN 496 | Positive | 35.26 |
| TP IRN 497 | Positive | 36.54 |
| TP IRN 498 | Positive | 31.95 |
| TP IRN 499 | Positive | 32.41 |
| TP IRN 500 | Positive | 33.51 |
| TP IRN 501 | Positive | 29.97 |
| TP IRN 502 | Negative | |
| TP IRN 503 | Positive | 34.85 |
| TP IRN 504 | Positive | 28.71 |
| TP IRN 505 | Positive | 31.19 |
| TP IRN 506 | Negative | |
| TP IRN 507 | Positive | 32.79 |
| TP IRN 508 | Positive | 32.26 |
| TP IRN 509 | Negative | |
| TP IRN 510 | Positive | 31.67 |
| TP IRN 511 | Positive | 33.24 |
| TP IRN 512 | Positive | 28.28 |
| TP IRN 513 | Positive | 28.73 |
| TP IRN 514 | Positive | 32.25 |
| TP IRN 515 | Positive | 32.52 |
| TP IRN 516 | Positive | 32.38 |
| TP IRN 517 | Positive | 36.22 |
| TP IRN 518 | Positive | 33.84 |
| TP IRN 519 | Positive | 30.48 |
| TP IRN 520 | Negative | |
| TP IRN 521 | Positive | 27.71 |
| TP IRN 522 | Negative | |
| TP IRN 523 | Positive | 32.85 |
| TP IRN 524 | Positive | 32.83 |
| TP IRN 525 | Positive | 31.42 |
| TP IRN 526 | Positive | 32.84 |
| TP IRN 527 | Positive | 36.96 |
| TP IRN 528 | Positive | 37.83 |
| TP IRN 529 | Positive | 33.96 |
| TP IRN 530 | Negative | |
| TP IRN 531 | Positive | 32.50 |
| TP IRN 532 | Positive | 33.80 |

| | | |
|---|---|---|
| TP IRN 533 | Positive | 32.47 |
| TP IRN 534 | Positive | 29.76 |
| TP IRN 535 | Positive | 32.82 |
| TP IRN 536 | Negative | |
| TP IRN 537 | Positive | 32.54 |
| TP IRN 538 | Negative | |
| TP IRN 539 | Negative | |
| TP IRN 540 | Negative | |
| TP IRN 541 | Positive | 34.81 |
| TP IRN 542 | Positive | 32.24 |
| TP IRN 543 | Positive | 31.71 |
| TP IRN 544 | Positive | 36.09 |
| TP IRN 545 | Positive | 31.63 |
| TP IRN_546 | Positive | 28.86 |
| TP IRN_547 | Positive | 32.60 |
| TP IRN_548 | Positive | 33.53 |
| TP IRN_549 | Positive | 33.76 |
| TP IRN_550 | Positive | 33.72 |
| TP IRN_551 | Positive | 31.28 |
| TP IRN_552 | Positive | 32.47 |
| TP IRN_553 | Positive | 32.70 |
| TP IRN_554 | Positive | 33.44 |
| TP IRN_555 | Positive | 32.83 |
| TP IRN_556 | Positive | 33.36 |
| TP IRN_557 | Positive | 32.40 |
| TP IRN_558 | Positive | 32.21 |
| TP IRN_559 | Positive | 35.60 |
| TP IRN_560 | Positive | 30.48 |
| TP IRN_561 | Positive | 32.83 |
| TP IRN_562 | Positive | 27.33 |
| TP IRN_563 | Positive | 32.30 |
| TP IRN_564 | Positive | 34.89 |
| TP IRN_565 | Negative | |
| TP IRN_566 | Positive | 32.42 |
| TP IRN_567 | Positive | 34.11 |
| TP IRN_568 | Positive | 32.10 |
| TP IRN_569 | Positive | 30.15 |
| TP IRN_570 | Positive | 32.01 |
| TP IRN_571 | Positive | 32.45 |
| TP IRN_572 | Positive | 33.95 |
| TP IRN_573 | Positive | 34.93 |
| TP IRN_574 | Positive | 36.06 |
| TP IRN_575 | Positive | 35.87 |
| TP IRN_576 | Positive | 36.49 |
| TP IRN_577 | Positive | 32.20 |
| TP IRN_578 | Positive | 33.27 |
| TP IRN_579 | Positive | 32.79 |
| TP IRN_580 | Positive | 34.60 |
| TP IRN_581 | Positive | 35.37 |

| | | |
|---|---|---|
| TP IRN_582 | Positive | 31.90 |
| TP IRN_583 | Positive | 27.73 |
| TP IRN_584 | Positive | 35.19 |
| TP IRN_585 | Positive | 32.90 |
| TP IRN_586 | Positive | 33.89 |
| TP IRN_587 | Positive | 36.14 |
| TP IRN_588 | Positive | 39.28 |
| TP IRN_589 | Positive | 35.08 |
| TP IRN_590 | Positive | 29.84 |
| TP IRN_591 | Positive | 34.03 |
| TP IRN_592 | Positive | 34.20 |
| TP IRN_593 | Positive | 32.49 |
| TP IRN_594 | Positive | 28.85 |
| TP IRN_595 | Positive | 30.94 |
| TP IRN_596 | Positive | 37.06 |
| TP IRN_597 | Positive | 33.81 |
| TP IRN_598 | Positive | 36.58 |
| TP IRN_599 | Negative | |
| TP IRN_600 | Positive | 30.49 |
| TP IRN_601 | Positive | 34.25 |
| TP IRN_602 | Positive | 30.70 |
| TP IRN_603 | Positive | 34.98 |
| TP IRN_604 | Positive | 33.56 |
| TP IRN_605 | Negative | |
| TP IRN_606 | Negative | |
| TP IRN_607 | Negative | |
| TP IRN_608 | Negative | |
| TP IRN_609 | Negative | |
| TP IRN_610 | Positive | 34.69 |
| TP IRN_611 | Negative | |
| TP IRN_612 | Positive | 30.36 |
| TP IRN_613 | Positive | 32.20 |
| TP IRN_614 | Positive | 33.58 |
| TP IRN_615 | Positive | 32.28 |
| TP IRN_616 | Positive | 34.02 |
| TP IRN_617 | Positive | 35.29 |
| TP IRN_618 | Positive | 32.73 |
| TP IRN_619 | Positive | 35.27 |
| TP IRN_620 | Positive | 25.94 |
| TP IRN_621 | Positive | 30.16 |
| TP IRN_622 | Positive | 30.85 |
| TP IRN_623 | Positive | 30.86 |
| TP IRN_624 | Positive | 31.22 |
| TP IRN_625 | Positive | 34.78 |
| TP IRN_626 | Positive | 30.66 |
| TP IRN_627 | Positive | 31.61 |
| TP IRN_628 | Positive | 29.96 |
| TP IRN_629 | Positive | 29.31 |
| TP IRN_630 | Positive | 37.09 |

| | | |
|---|---|---|
| TP IRN_631 | Negative | |
| TP IRN_632 | Positive | 30.73 |
| TP IRN_633 | Positive | 25.77 |
| TP IRN_634 | Positive | 28.26 |
| TP IRN_635 | Positive | 32.13 |
| TP IRN_636 | Positive | 29.94 |
| TP IRN_637 | Positive | 32.91 |
| TP IRN_638 | Positive | 29.81 |
| TP IRN_639 | Positive | 32.44 |
| TP IRN_640 | Positive | 32.67 |
| TP IRN_641 | Positive | 29.24 |
| TP IRN_642 | Positive | 30.71 |
| TP IRN_643 | Positive | 32.45 |
| TP IRN_644 | Positive | 31.87 |
| TP IRN_645 | Positive | 33.27 |
| TP IRN_646 | Positive | 34.14 |
| TP IRN_647 | Positive | 32.84 |
| TP IRN_648 | Positive | 31.49 |
| TP IRN_649 | Positive | 34.29 |
| TP IRN_650 | Positive | 34.86 |
| TP IRN_651 | Positive | 29.67 |
| TP IRN_652 | Positive | 29.66 |
| TP IRN_653 | Positive | 36.27 |
| TP IRN_654 | Positive | 32.98 |
| TP IRN_655 | Negative | |
| TP IRN_656 | Negative | |
| TP IRN_657 | Positive | 38.99 |
| TP IRN_658 | Positive | 33.50 |
| TP IRN_659 | Negative | |
| TP IRN_660 | Positive | 32.85 |
| TP IRN_661 | Positive | 35.39 |
| TP IRN_662 | Positive | 31.91 |
| TP IRN_663 | Positive | 34.95 |
| TP IRN_664 | Positive | 33.21 |
| TP IRN_665 | Negative | |
| TP IRN_666 | Negative | |
| TP IRN_667 | Positive | 31.48 |
| TP IRN_668 | Positive | 32.37 |
| TP IRN_669 | Positive | 34.81 |
| TP IRN_670 | Positive | 27.62 |
| TP IRN_671 | Negative | |
| TP IRN_672 | Negative | |
| TP IRN_673 | Positive | 33.28 |
| TP IRN_674 | Negative | |
| TP IRN_675 | Positive | 34.66 |
| TP IRN_676 | Positive | 33.21 |
| TP IRN_677 | Negative | |
| TP IRN_678 | Positive | 30.82 |
| TP IRN_679 | Negative | |

| | | |
|---|---|---|
| TP IRN_680 | Negative | |
| TP IRN_681 | Positive | 32.97 |
| TP IRN_682 | Negative | |
| TP IRN_683 | Negative | |
| TP IRN_684 | Positive | 35.50 |
| TP IRN_685 | Negative | |
| TP IRN_686 | Positive | 33.54 |
| TP IRN_687 | Positive | 31.37 |
| TP IRN_688 | Negative | |
| TP IRN_689 | Positive | 26.77 |
| TP IRN_690 | Negative | |
| TP IRN_691 | Positive | 31.85 |
| TP IRN_692 | Positive | 31.44 |
| TP IRN_693 | Negative | |
| TP IRN_694 | Positive | 30.59 |
| TP IRN_695 | Positive | 32.00 |
| TP IRN_696 | Positive | 31.51 |
| TP IRN_697 | Positive | 28.78 |
| TP IRN_698 | Positive | 29.71 |
| TP IRN_699 | Positive | 32.45 |
| TP IRN_700 | Positive | 30.91 |
| TP IRN_701 | Positive | 30.41 |
| TP IRN_702 | Negative | |
| TP IRN_703 | Positive | 33.73 |
| TP IRN_704 | Negative | |
| TP IRN_705 | Positive | 32.51 |
| TP IRN_706 | Positive | 31.37 |
| TP IRN_707 | Positive | 32.76 |
| TP IRN_708 | Negative | |
| TP IRN_709 | Positive | 31.82 |
| TP IRN_710 | Positive | 31.22 |
| TP IRN_711 | Positive | 29.96 |
| TP IRN_712 | Negative | |
| TP IRN_713 | Negative | |
| TP IRN_714 | Negative | |
| TP IRN_715 | Positive | 28.84 |
| TP IRN_716 | Positive | 29.23 |
| TP IRN_717 | Positive | 32.90 |
| TP IRN_718 | Positive | 33.17 |
| TP IRN_719 | Positive | 30.11 |
| TP IRN_720 | Positive | 30.19 |
| TP IRN_721 | Positive | 29.80 |
| TP IRN_722 | Positive | 35.73 |
| TP IRN_723 | Negative | |
| TP IRN_724 | Positive | 31.21 |
| TP IRN_725 | Positive | 33.94 |
| TP IRN_726 | Positive | 32.33 |
| TP IRN_727 | Positive | 31.11 |
| TP IRN_728 | Positive | 32.71 |

| | | |
|---|---|---|
| TP IRN_729 | Positive | 30.47 |
| TP IRN_730 | Positive | 33.82 |
| TP IRN_731 | Negative | |
| TP IRN_732 | Positive | 33.33 |
| TP IRN_733 | Positive | 32.76 |
| TP IRN_734 | Positive | 31.20 |
| TP IRN_735 | Positive | 30.05 |
| TP IRN_736 | Positive | 32.20 |
| TP IRN_737 | Positive | 33.66 |
| TP IRN_738 | Positive | 31.22 |
| TP IRN_739 | Positive | 32.57 |
| TP IRN_740 | Positive | 29.64 |
| TP IRN_741 | Positive | 33.70 |
| TP IRN_742 | Positive | 33.20 |
| TP IRN_743 | Negative | |
| TP IRN_744 | Negative | |
| TP IRN_745 | Positive | 32.40 |
| TP IRN_746 | Positive | 26.99 |
| TP IRN_747 | Positive | 31.48 |
| TP IRN_748 | Positive | 33.82 |
| TP IRN_749 | Positive | 31.74 |
| TP IRN_750 | Positive | 33.76 |
| TP IRN_751 | Negative | |
| TP IRN_752 | Negative | |
| TP IRN_753 | Negative | |
| TP IRN_754 | Negative | |
| TP IRN_755 | Positive | 32.65 |
| TP IRN_756 | Positive | 29.15 |
| TP IRN_757 | Negative | |
| TP IRN_758 | Positive | 30.64 |
| TP IRN_759 | Positive | 31.42 |
| TP IRN_760 | Positive | 34.58 |
| TP IRN_761 | Negative | |
| TP IRN_762 | Positive | 33.77 |
| TP IRN_763 | Positive | 31.89 |
| TP IRN_764 | Negative | |
| TP IRN_765 | Negative | |
| TP IRN_766 | Positive | 32.85 |
| TP IRN_767 | Positive | 31.76 |
| TP IRN_768 | Positive | 31.73 |
| TP IRN_769 | Positive | 36.65 |
| TP IRN_770 | Positive | 31.60 |
| TP IRN_771 | Positive | 30.60 |
| TP IRN_772 | Positive | 31.61 |
| TP IRN_773 | Positive | 34.15 |
| TP IRN_774 | Positive | 32.22 |
| TP IRN_775 | Negative | |
| TP IRN_776 | Positive | 30.69 |
| TP IRN_777 | Negative | |

| | | |
|---|---|---|
| TP IRN_778 | Positive | 30.86 |
| TP IRN_779 | Negative | |
| TP IRN_780 | Positive | 31.25 |
| TP IRN_781 | Positive | 29.40 |
| TP IRN_782 | Positive | 31.78 |
| TP IRN_783 | Positive | 30.74 |
| TP IRN_784 | Positive | 29.77 |
| TP IRN_785 | Negative | |
| TP IRN_786 | Positive | 30.50 |
| TP IRN_787 | Negative | |
| TP IRN_788 | Positive | 30.73 |
| TP IRN_789 | Positive | 31.47 |
| TP IRN_790 | Negative | |
| TP IRN_791 | Negative | |
| TP IRN_792 | Positive | 31.91 |
| TP IRN_793 | Positive | 35.57 |
| TP IRN_794 | Positive | 24.00 |
| TP IRN_795 | Negative | |
| TP IRN_796 | Positive | 33.45 |
| TP IRN_797 | Negative | |
| TP IRN_798 | Negative | |
| TP IRN_799 | Positive | 34.11 |
| TP IRN_800 | Negative | |
| TP IRN_801 | Negative | |
| TP IRN_802 | Negative | |
| TP IRN_803 | Positive | 30.06 |
| TP IRN_804 | Negative | |
| TP IRN_805 | Positive | 34.81 |
| TP IRN_806 | Negative | |
| TP IRN_807 | Negative | |
| TP IRN_808 | Positive | 37.56 |
| TP IRN_809 | Negative | |
| TP IRN_810 | Positive | 31.72 |
| TP IRN_811 | Positive | 33.52 |
| TP IRN_812 | Positive | 35.18 |
| TP IRN_813 | Negative | |
| TP IRN_814 | Negative | |
| TP IRN_815 | Negative | |
| TP IRN_816 | Positive | 33.55 |
| TP IRN_817 | Positive | 30.32 |
| TP IRN_818 | Negative | |
| TP IRN_819 | Positive | 32.81 |
| TP IRN_820 | Positive | 33.17 |
| TP IRN_821 | Negative | |
| TP IRN_822 | Negative | |
| TP IRN_823 | Positive | 35.59 |
| TP IRN_824 | Negative | |
| TP IRN_825 | Positive | 32.28 |
| TP IRN_826 | Negative | |

| | | |
|---|---|---|
| TP IRN_827 | Negative | |
| TP IRN_828 | Negative | |
| TP IRN_829 | Positive | 33.03 |
| TP IRN_830 | Negative | |
| TP IRN_831 | Positive | 34.32 |
| TP IRN_832 | Positive | 34.65 |
| TP IRN_833 | Negative | |
| TP IRN_834 | Negative | |
| TP IRN_835 | Negative | |
| TP IRN_836 | Positive | 33.11 |
| TP IRN_837 | Positive | 31.59 |
| TP IRN_838 | Negative | |
| TP IRN_839 | Negative | |
| TP IRN_840 | Positive | 34.49 |
| TP IRN_841 | Positive | 32.26 |
| TP IRN_842 | Positive | 32.66 |
| TP IRN_843 | Positive | 33.32 |
| TP IRN_844 | Negative | |
| TP IRN_845 | Positive | 31.86 |
| TP IRN_846 | Positive | 35.34 |
| TP IRN_847 | Positive | 34.64 |
| TP IRN_848 | Positive | 31.24 |
| TP IRN_849 | Positive | 32.98 |
| TP IRN_850 | Negative | |
| TP IRN_851 | Negative | |
| TP IRN_852 | Positive | 33.26 |
| TP IRN_853 | Negative | |
| TP IRN_854 | Positive | 33.59 |
| TP IRN_855 | Positive | 34.26 |
| TP IRN_856 | Negative | |
| TP IRN_857 | Positive | 34.30 |
| TP IRN_858 | Positive | 38.29 |
| TP IRN_859 | Positive | 32.92 |
| TP IRN_860 | Positive | 33.78 |
| TP IRN_861 | Positive | 33.19 |
| TP IRN_862 | Positive | 27.70 |
| TP IRN_863 | Positive | 31.49 |
| TP IRN_864 | Positive | 32.72 |
| TP IRN_865 | Positive | 33.59 |
| TP IRN_866 | Positive | 32.86 |
| TP IRN_867 | Positive | 37.56 |
| TP IRN_868 | Positive | 29.82 |
| TP IRN_869 | Positive | 31.82 |
| TP IRN_870 | Negative | |
| TP IRN_871 | Negative | |
| TP IRN_872 | Positive | 28.59 |
| TP IRN_873 | Negative | |
| TP IRN_874 | Positive | 32.84 |
| TP IRN_875 | Positive | 31.81 |

| | | |
|---|---|---|
| TP IRN_876 | Positive | 31.33 |
| TP IRN_877 | Negative | |
| TP IRN_878 | Negative | |
| TP IRN_879 | Positive | 31.25 |
| TP IRN_880 | Negative | |
| TP IRN_881 | Positive | 31.57 |
| TP IRN_882 | Positive | 33.58 |
| TP IRN_883 | Negative | |
| TP IRN_884 | Positive | 31.47 |
| TP IRN_885 | Positive | 31.94 |
| TP IRN_886 | Positive | 30.66 |
| TP IRN_887 | Positive | 28.71 |
| TP IRN_888 | Positive | 32.86 |
| TP IRN_889 | Positive | 32.60 |
| TP IRN_890 | Positive | 32.51 |
| TP IRN_891 | Positive | 31.62 |
| TP IRN_892 | Positive | 32.74 |
| TP IRN_893 | Positive | 31.37 |
| TP IRN_894 | Positive | 32.46 |
| TP IRN_895 | Positive | 31.32 |
| TP IRN_896 | Positive | 32.32 |
| TP IRN_897 | Positive | 32.09 |
| TP IRN_898 | Positive | 30.86 |
| TP IRN_899 | Positive | 31.16 |
| TP IRN_900 | Negative | |
| TP IRN_901 | Negative | |
| TP IRN_902 | Positive | 30.87 |
| TP IRN_903 | Positive | 33.65 |
| TP IRN_904 | Positive | 32.81 |
| TP IRN_905 | Negative | |
| TP IRN_906 | Positive | 33.16 |
| TP IRN_907 | Positive | 33.48 |
| TP IRN_908 | Positive | 31.72 |
| TP IRN_909 | Negative | |
| TP IRN_910 | Positive | 33.61 |
| TP IRN_911 | Positive | 33.97 |
| TP IRN_912 | Negative | |
| TP IRN_913 | Positive | 33.16 |
| TP IRN_914 | Positive | 33.01 |
| TP IRN_915 | Positive | 37.81 |
| TP IRN_916 | Positive | 29.76 |
| TP IRN_917 | Positive | 32.44 |
| TP IRN_918 | Negative | |
| TP IRN_919 | Positive | 32.69 |
| TP IRN_920 | Negative | |
| TP IRN_921 | Positive | 33.81 |
| TP IRN_922 | Positive | 31.68 |
| TP IRN_923 | Positive | 32.94 |
| TP IRN_924 | Positive | 30.12 |

| | | |
|---|---|---|
| TP IRN_925 | Positive | 35.84 |
| TP IRN_926 | Positive | 33.04 |
| TP IRN_927 | Positive | 33.60 |
| TP IRN_928 | Positive | 32.90 |
| TP IRN_929 | Negative | |
| TP IRN_930 | Positive | 31.80 |
| TP IRN_931 | Positive | 31.53 |
| TP IRN_932 | Positive | 33.30 |
| TP IRN_933 | Positive | 37.10 |
| TP IRN_934 | Positive | 30.75 |
| TP IRN_935 | Negative | |
| TP IRN_936 | Positive | 31.40 |
| TP IRN_937 | Negative | |
| TP IRN_938 | Positive | 31.59 |
| TP IRN_939 | Negative | |
| TP IRN_940 | Positive | 31.15 |
| TP IRN_941 | Positive | 33.72 |
| TP IRN_942 | Positive | 32.34 |
| TP IRN_943 | Negative | |
| TP IRN_944 | Negative | |
| TP IRN_945 | Negative | |
| TP IRN_946 | Negative | |
| TP IRN_947 | Positive | 32.86 |
| TP IRN_948 | Positive | 32.55 |
| TP IRN_949 | Positive | 32.74 |
| TP IRN_950 | Positive | 33.24 |
| TP IRN_951 | Positive | 31.70 |
| TP IRN_952 | Positive | 31.84 |
| TP IRN_953 | Positive | 31.13 |
| TP IRN_954 | Positive | 34.08 |
| TP IRN_955 | Positive | 32.91 |
| TP IRN_956 | Positive | 31.51 |
| TP IRN_957 | Positive | 33.72 |
| TP IRN_958 | Positive | 34.68 |
| TP IRN_959 | Positive | 34.78 |
| TP IRN_960 | Positive | 33.16 |
| TP IRN_961 | Positive | 32.41 |
| TP IRN_962 | Positive | 33.70 |
| TP IRN_963 | Positive | 33.37 |
| TP IRN_964 | Positive | 31.96 |
| TP IRN_965 | Positive | 34.28 |
| TP IRN_966 | Positive | 32.87 |
| TP IRN_967 | Positive | 31.46 |
| TP IRN_968 | Positive | 28.96 |
| TP IRN_969 | Positive | 31.88 |
| TP IRN_970 | Positive | 32.37 |
| TP IRN_971 | Positive | 33.99 |
| TP IRN_972 | Positive | 35.66 |
| TP IRN_973 | Positive | 32.70 |

| | | |
|---|---|---|
| TP IRN_974 | Negative | |
| TP IRN_975 | Positive | 32.94 |
| TP IRN_976 | Positive | 32.54 |
| TP IRN_977 | Negative | |
| TP IRN_978 | Positive | 32.19 |
| TP IRN_979 | Positive | 33.99 |
| TP IRN_980 | Positive | 32.94 |
| TP IRN_981 | Positive | 30.21 |
| TP IRN_982 | Positive | 31.09 |
| TP IRN_983 | Positive | 30.49 |
| TP IRN_984 | Positive | 33.53 |
| TP IRN_985 | Positive | 31.37 |
| TP IRN_986 | Positive | 38.22 |
| TP IRN_987 | Positive | 31.90 |
| TP IRN_988 | Negative | |
| TP IRN_989 | Negative | |
| TP IRN_990 | Positive | 31.84 |
| TP IRN_991 | Positive | 32.40 |
| TP IRN_992 | Positive | 32.39 |
| TP IRN_993 | Positive | 33.40 |
| TP IRN_994 | Positive | 34.14 |
| TP IRN_995 | Positive | 32.84 |
| TP IRN_996 | Positive | 31.98 |
| TP IRN_997 | Positive | 32.58 |
| TP IRN_998 | Positive | 31.98 |
| TP IRN_999 | Positive | 35.52 |
| TP IRN_1000 | Positive | 32.83 |
| TP IRN_1001 | Positive | 32.24 |
| TP IRN_1002 | Positive | 32.23 |
| TP IRN_1003 | Negative | |
| TP IRN_1004 | Positive | 31.59 |
| TP IRN_1005 | Positive | 30.44 |
| TP IRN_1006 | Positive | 33.11 |
| TP IRN_1007 | Positive | 28.84 |
| TP IRN_1008 | Positive | 27.45 |
| TP IRN_1009 | Positive | 35.52 |
| TP IRN_1010 | Positive | 28.68 |
| TP IRN_1011 | Positive | 34.53 |
| TP IRN_1012 | Positive | 31.37 |
| TP IRN_1013 | Positive | 28.60 |
| TP IRN_1014 | Positive | 31.39 |
| TP IRN_1015 | Positive | 28.99 |
| TP IRN_1016 | Positive | 32.60 |
| TP IRN_1017 | Positive | 27.43 |
| TP IRN_1018 | Positive | 28.73 |
| TP IRN_1019 | Positive | 30.89 |
| TP IRN_1020 | Positive | 29.84 |
| TP IRN_1021 | Positive | 32.21 |
| TP IRN_1022 | Positive | 32.90 |

| | | |
|---|---|---|
| TP IRN_1023 | Positive | 34.61 |
| TP IRN_1024 | Negative | |
| TP IRN_1025 | Positive | 30.61 |
| TP IRN_1026 | Positive | 29.60 |
| TP IRN_1027 | Positive | 34.92 |
| TP IRN_1028 | Positive | 27.52 |
| TP IRN_1029 | Positive | 31.43 |
| TP IRN_1030 | Positive | 26.98 |
| TP IRN_1031 | Positive | 30.79 |
| TP IRN_1032 | Positive | 33.41 |
| TP IRN_1033 | Positive | 31.37 |
| TP IRN_1034 | Negative | |
| TP IRN_1035 | Positive | 34.74 |
| TP IRN_1036 | Positive | 35.75 |
| TP IRN_1037 | Positive | 32.01 |
| TP IRN_1038 | Positive | 32.46 |
| TP IRN_1039 | Negative | |
| TP IRN_1040 | Positive | 36.90 |
| TP IRN_1041 | Negative | |
| TP IRN_1042 | Negative | |
| TP IRN_1043 | Negative | |
| TP IRN_1044 | Positive | 31.54 |
| TP IRN_1045 | Positive | 32.73 |
| TP IRN_1046 | Positive | 34.35 |
| TP IRN_1047 | Positive | 30.47 |
| TP IRN_1048 | Positive | 35.35 |
| TP IRN_1049 | Negative | |
| TP IRN_1050 | Positive | 33.08 |
| TP IRN_1051 | Positive | 32.50 |
| TP IRN_1052 | Positive | 32.26 |
| TP IRN_1053 | Positive | 27.59 |
| TP IRN_1054 | Negative | |
| TP IRN_1055 | Positive | 29.50 |
| TP IRN_1056 | Negative | |
| TP IRN_1057 | Positive | 30.47 |
| TP IRN_1058 | Positive | 31.23 |
| TP IRN_1059 | Positive | 33.37 |
| TP IRN_1060 | Negative | |
| TP IRN_1061 | Positive | 33.18 |
| TP IRN_1062 | Positive | 34.28 |
| TP IRN_1063 | Positive | 33.21 |
| TP IRN_1064 | Positive | 28.52 |
| TP IRN_1065 | Positive | 30.88 |
| TP IRN_1066 | Positive | 32.16 |
| TP IRN_1067 | Positive | 34.15 |
| TP IRN_1068 | Positive | 25.35 |
| TP IRN_1069 | Positive | 28.67 |
| TP IRN_1070 | Positive | 33.88 |
| TP IRN_1071 | Positive | 28.18 |

| | | |
|---|---|---|
| TP IRN_1072 | Negative | |
| TP IRN_1073 | Positive | 29.84 |
| TP IRN_1074 | Negative | |
| TP IRN_1075 | Positive | 30.22 |
| TP IRN_1076 | Positive | 28.35 |
| TP IRN_1077 | Positive | 29.02 |
| TP IRN_1078 | Negative | |
| TP IRN_1079 | Positive | 36.23 |
| TP IRN_1080 | Negative | |
| TP IRN_1081 | Positive | 33.38 |
| TP IRN_1082 | Negative | |
| TP IRN_1083 | Negative | |
| TP IRN_1084 | Positive | 34.12 |
| TP IRN_1085 | Positive | 29.52 |
| TP IRN_1086 | Positive | 29.87 |
| TP IRN_1087 | Positive | 31.34 |
| TP IRN_1088 | Positive | 33.97 |
| TP IRN_1089 | Negative | |
| TP IRN_1090 | Negative | |
| TP IRN_1091 | Positive | 30.01 |
| TP IRN_1092 | Positive | 35.00, |
| TP IRN_1093 | Positive | 29.44 |
| TP IRN_1094 | Negative | |
| TP IRN_1095 | Negative | |
| TP IRN_1096 | Positive | 32.84 |
| TP IRN_1097 | Negative | |
| TP IRN_1098 | Negative | |
| TP IRN_1099 | Positive | 31.92 |
| TP IRN_1100 | Positive | 31.14 |
| TP IRN_1101 | Positive | 30.10 |
| TP IRN_1102 | Negative | |
| TP IRN_1103 | Negative | |
| TP IRN_1104 | Positive | 33.76 |
| TP IRN_1105 | Positive | 30.86 |
| TP IRN_1106 | Positive | 32.25 |
| TP IRN_1107 | Negative | |
| TP IRN_1108 | Positive | 35.69 |
| TP IRN_1109 | Positive | 34.25 |
| TP IRN_1110 | Negative | |
| TP IRN_1111 | Positive | 34.54 |
| TP IRN_1112 | Positive | 34.93 |
| TP IRN_1113 | Positive | 31.38 |
| TP IRN_1114 | Positive | 36.72 |
| TP IRN_1115 | Positive | 34.48 |
| TP IRN_1116 | Positive | 32.74 |
| TP IRN_1117 | Positive | 35.39 |
| TP IRN_1118 | Positive | 34.52 |
| TP IRN_1119 | Positive | 34.39 |
| TP IRN_1120 | Negative | |

| | | |
|---|---|---|
| TP IRN_1121 | Negative | |
| TP IRN_1122 | Negative | |
| TP IRN_1123 | Positive | 33.81 |
| TP IRN_1124 | Negative | |
| TP IRN_1125 | Positive | 34.59 |
| TP IRN_1126 | Positive | 30.94 |
| TP IRN_1127 | Negative | |
| TP IRN_1128 | Positive | 30.50 |
| TP IRN_1129 | Positive | 32.16 |
| TP IRN_1130 | Positive | 31.05 |
| TP IRN_1131 | Negative | |
| TP IRN_1132 | Positive | 29.89 |
| TP IRN_1133 | Positive | 28.97 |
| TP IRN_1134 | Positive | 28.53 |
| TP IRN_1135 | Positive | 27.86 |
| TP IRN_1136 | Positive | 27.79 |
| TP IRN_1137 | Positive | 31.25 |
| TP IRN_1138 | Positive | 28.68 |
| TP IRN_1139 | Positive | 28.15 |
| TP IRN_1140 | Positive | 27.51 |
| TP IRN_1141 | Positive | 28.29 |
| TP IRN_1142 | Positive | 32.73 |
| TP IRN_1143 | Negative | |
| TP IRN_1144 | Positive | 31.74 |
| TP IRN_1145 | Positive | 27.95 |
| TP IRN_1146 | Positive | 30.16 |
| TP IRN_1147 | Positive | 27.98 |
| TP IRN_1148 | Positive | 27.06 |
| TP IRN_1149 | Positive | 25.57 |
| TP IRN_1150 | Positive | 28.19 |
| TP IRN_1151 | Positive | 25.22 |
| TP IRN_1152 | Positive | 25.59 |
| TP IRN_1153 | Positive | 26.47 |
| TP IRN_1154 | Positive | 25.00 |
| TP IRN_1155 | Positive | 25.23 |
| TP IRN_1156 | Positive | 25.35 |
| TP IRN_1157 | Positive | 25.50 |
| TP IRN_1158 | Positive | 25.50 |
| TP IRN_1159 | Positive | 33.30 |
| TP IRN_1160 | Positive | 24.95 |
| TP IRN_1161 | Positive | 24.28 |
| TP IRN_1162 | Positive | 25.63 |
| TP IRN_1163 | Positive | 25.37 |
| TP IRN_1164 | Negative | |
| TP IRN_1165 | Positive | 24.50 |
| TP IRN_1166 | Positive | 26.55 |
| TP IRN_1167 | Positive | 30.73 |
| TP IRN_1168 | Positive | 25.25 |
| TP IRN_1169 | Positive | 26.26 |

| | | |
|---|---|---|
| TP IRN_1170 | Positive | 23.86 |
| TP IRN_1171 | Positive | 25.58 |
| TP IRN_1172 | Negative | |
| TP IRN_1173 | Positive | 24.14 |
| TP IRN_1174 | Positive | 25.71 |
| TP IRN_1175 | Negative | |
| TP IRN_1176 | Positive | 33.46 |
| TP IRN_1177 | Negative | |
| TP IRN_1178 | Positive | 32.42 |
| TP IRN_1179 | Positive | 33.93 |
| TP IRN_1180 | Positive | 36.33 |
| TP IRN_1181 | Positive | 32.93 |
| TP IRN_1182 | Positive | 32.57 |
| TP IRN_1183 | Negative | |
| TP IRN_1184 | Positive | 34.34 |
| TP IRN_1185 | Negative | |
| TP IRN_1186 | Negative | |
| TP IRN_1187 | Negative | |
| TP IRN_1188 | Negative | |
| TP IRN_1189 | Negative | |
| TP IRN_1190 | Negative | |
| TP IRN_1191 | Positive | 32.95 |
| TP IRN_1192 | Negative | |
| TP IRN_1193 | Positive | 32.27 |
| TP IRN_1194 | Positive | 36.86 |
| TP IRN_1195 | Positive | 33.92 |
| TP IRN_1196 | Negative | |
| TP IRN_1197 | Positive | 31.57 |
| TP IRN_1198 | Negative | |
| TP IRN_1199 | Negative | |
| TP IRN_1200 | Negative | |
| TP IRN_1201 | Positive | 32.97 |
| TP IRN_1202 | Negative | |
| TP IRN_1203 | Positive | 31.94 |
| TP IRN_1204 | Positive | 33.59 |
| TP IRN_1205 | Positive | 33.35 |
| TP IRN_1206 | Positive | 33.84 |
| TP IRN_1207 | Positive | 33.08 |
| TP IRN_1208 | Positive | 32.74 |
| TP IRN_1209 | Negative | |
| TP IRN_1210 | Negative | |
| TP IRN_1211 | Negative | |
| TP IRN_1212 | Negative | |
| TP IRN_1213 | Negative | |
| TP IRN_1214 | Positive | 37.45 |
| TP IRN_1215 | Negative | |
| TP IRN_1216 | Positive | 37.55 |
| TP IRN_1217 | Negative | |
| TP IRN_1218 | Negative | |

| | | |
|---|---|---|
| TP IRN_1219 | Negative | |
| TP IRN_1220 | Positive | 30.53 |
| TP IRN_1221 | Positive | 33.42 |
| TP IRN_1222 | Negative | |
| TP IRN_1223 | Negative | |
| TP IRN_1224 | Positive | 33.75 |
| TP IRN_1225 | Positive | 33.02 |
| TP IRN_1226 | Negative | |
| TP IRN_1227 | Negative | |
| TP IRN_1228 | Negative | |
| TP IRN_1229 | Negative | |
| TP IRN_1230 | Positive | 33.09 |
| TP IRN_1231 | Negative | |
| TP IRN_1232 | Positive | 36.67 |
| TP IRN_1233 | Negative | |
| TP IRN_1234 | Negative | |
| TP IRN_1235 | Positive | 31.23 |
| TP IRN_1236 | Positive | 33.51 |
| TP IRN_1237 | Negative | |
| TP IRN_1238 | Positive | 31.41 |
| TP IRN_1239 | Positive | 34.80 |
| TP IRN_1240 | Negative | |
| TP IRN_1241 | Negative | |
| TP IRN_1242 | Negative | |
| TP IRN_1243 | Negative | |
| TP IRN_1244 | Negative | |
| TP IRN_1245 | Positive | 37.07 |
| TP IRN_1246 | Negative | |
| TP IRN_1247 | Negative | |
| TP IRN_1248 | Negative | |
| TP IRN_1249 | Negative | |
| TP IRN_1250 | Positive | 32.13 |
| TP IRN_1251 | Negative | |
| TP IRN_1252 | Positive | 33.55 |
| TP IRN_1253 | Positive | 37.12 |
| TP IRN_1254 | Negative | |
| TP IRN_1255 | Positive | 34.12 |
| TP IRN_1256 | Negative | |
| TP IRN_1257 | Negative | |
| TP IRN_1258 | Negative | |
| TP IRN_1259 | Negative | |
| TP IRN_1260 | Positive | 35.88 |
| TP IRN_1261 | Negative | |
| TP IRN_1262 | Negative | |
| TP IRN_1263 | Negative | |
| TP IRN_1264 | Negative | |
| TP IRN_1265 | Positive | 33.39 |
| TP IRN_1266 | Negative | |
| TP IRN_1267 | Negative | |

| | | |
|---|---|---|
| TP IRN_1268 | Negative | |
| TP IRN_1269 | Negative | |
| TP IRN_1270 | Positive | 31.93 |
| TP IRN_1271 | Negative | |
| TP IRN_1272 | Negative | |
| TP IRN_1273 | Negative | |
| TP IRN_1274 | Positive | 31.94 |
| TP IRN_1275 | Positive | 35.42 |
| TP IRN_1276 | Negative | |
| TP IRN_1277 | Positive | 32.04 |
| TP IRN_1278 | Negative | |
| TP IRN_1279 | Positive | 32.94 |
| TP IRN_1280 | Positive | 26.67 |
| TP IRN_1281 | Negative | |
| TP IRN_1282 | Negative | |
| TP IRN_1283 | Positive | 32.55 |
| TP IRN_1284 | Negative | |
| TP IRN_1285 | Negative | |
| TP IRN_1286 | Negative | |
| TP IRN_1287 | Positive | 33.88 |
| TP IRN_1288 | Negative | |
| TP IRN_1289 | Negative | |
| TP IRN_1290 | Positive | 37.15 |
| TP IRN_1291 | Negative | |
| TP IRN_1292 | Positive | 36.03 |
| TP IRN_1293 | Positive | 34.15 |
| TP IRN_1294 | Negative | |
| TP IRN_1295 | Negative | |
| TP IRN_1296 | Positive | 33.63 |
| TP IRN_1297 | Positive | 33.84 |
| TP IRN_1298 | Negative | |
| TP IRN_1299 | Positive | 33.58 |
| TP IRN_1300 | Negative | |
| TP IRN_1301 | Negative | |
| TP IRN_1302 | Positive | 33.64 |
| TP IRN_1303 | Positive | 32.77 |
| TP IRN_1304 | Positive | 33.64 |
| TP IRN_1305 | Negative | |
| TP IRN_1306 | Negative | |
| TP IRN_1307 | Positive | 34.67 |
| TP IRN_1308 | Negative | |
| TP IRN_1309 | Positive | 32.75 |
| TP IRN_1310 | Positive | 31.16 |
| TP IRN_1311 | Negative | |
| TP IRN_1312 | Positive | 33.68 |
| TP IRN_1313 | Negative | |
| TP IRN_1314 | Negative | |
| TP IRN_1315 | Negative | |
| TP IRN_1316 | Negative | |

| | | |
|---|---|---|
| TP IRN_1317 | Positive | 34.31 |
| TP IRN_1318 | Positive | 32.84 |
| TP IRN_1319 | Negative | |
| TP IRN_1320 | Positive | 34.56 |
| TP IRN_1321 | Negative | |
| TP IRN_1322 | Positive | 35.96 |
| TP IRN_1323 | Positive | 31.50 |
| TP IRN_1324 | Negative | |
| TP IRN_1325 | Positive | 33.74 |
| TP IRN_1326 | Positive | 33.19 |
| TP IRN_1327 | Negative | |
| TP IRN_1328 | Positive | 31.34 |
| TP IRN_1329 | Negative | |
| TP IRN_1330 | Positive | 33.56 |
| TP IRN_1331 | Negative | |
| TP IRN_1332 | Negative | |
| TP IRN_1333 | Negative | |
| TP IRN_1334 | Negative | |
| TP IRN_1335 | Positive | 33.85 |
| TP IRN_1336 | Negative | |
| TP IRN_1337 | Negative | |
| TP IRN_1338 | Negative | |
| TP IRN_1339 | Negative | |
| TP IRN_1340 | Negative | |
| TP IRN_1341 | Positive | 35.03 |
| TP IRN_1342 | Negative | |
| TP IRN_1343 | Positive | 20.00 |
| TP IRN_1344 | Negative | |
| TP IRN_1345 | Positive | 31.57 |
| TP IRN_1346 | Positive | 34.09 |
| TP IRN_1347 | Negative | |
| TP IRN_1348 | Positive | 33.29 |
| TP IRN_1349 | Positive | 32.33 |
| TP IRN_1350 | Negative | |
| TP IRN_1351 | Negative | |
| TP IRN_1352 | Positive | 35.68 |
| TP IRN_1353 | Negative | |
| TP IRN_1354 | Negative | |
| TP IRN_1355 | Negative | |
| TP IRN_1356 | Negative | |
| TP IRN_1357 | Positive | 32.07 |
| TP IRN_1358 | Negative | |
| TP IRN_1359 | Negative | |
| TP IRN_1360 | Positive | 33.96 |
| TP IRN_1361 | Negative | |
| TP IRN_1362 | Negative | |
| TP IRN_1363 | Negative | |
| TP IRN_1364 | Negative | |
| TP IRN_1365 | Negative | |

| | |
|---|---|
| TP IRN_1366 | Negative |
| TP IRN_1367 | Negative |
| TP IRN_1368 | Negative |
| TP IRN_1369 | Negative |
| TP IRN_1370 | Negative |
| TP IRN_1371 | Negative |
| TP IRN_1372 | Negative |
| TP IRN_1373 | Negative |
| TP IRN_1374 | Negative |
| TP IRN_1375 | Negative |
| TP IRN_1376 | Negative |
| TP IRN_1377 | Negative |
| TP IRN_1378 | Negative |
| TP IRN_1379 | Negative |
| TP IRN_1380 | Negative |
| TP IRN_1381 | Negative |
| TP IRN_1382 | Negative |
| TP IRN_1383 | Negative |
| TP IRN_1384 | Negative |
| TP IRN_1385 | Negative |
| TP IRN_1386 | Negative |
| TP IRN_1387 | Negative |
| TP IRN_1388 | Negative |
| TP IRN_1389 | Negative |
| TP IRN_1390 | Negative |
| TP IRN_1391 | Negative |
| TP IRN_1392 | Negative |
| TP IRN_1393 | Negative |
| TP IRN_1394 | Negative |
| TP IRN_1395 | Negative |
| TP IRN_1396 | Negative |
| TP IRN_1397 | Negative |
| TP IRN_1398 | Negative |
| TP IRN_1399 | Negative |
| TP IRN_1400 | Negative |
| TP IRN_1401 | Negative |
| TP IRN_1402 | Negative |
| TP IRN_1403 | Negative |
| TP IRN_1404 | Negative |
| TP IRN_1405 | Negative |
| TP IRN_1406 | Negative |
| TP IRN_1407 | Negative |
| TP IRN_1408 | Negative |
| TP IRN_1409 | Negative |
| TP IRN_1410 | Negative |
| TP IRN_1411 | Negative |
| TP IRN_1412 | Negative |
| TP IRN_1413 | Negative |
| TP IRN_1414 | Negative |

| | | |
|---|---|---|
| TP IRN_1415 | Negative | |
| TP IRN_1416 | Negative | |
| TP IRN_1417 | Negative | |
| TP IRN_1418 | Negative | |
| TP IRN_1419 | Negative | |
| TP IRN_1420 | Negative | |
| TP IRN_1421 | Negative | |
| TP IRN_1422 | Negative | |
| TP IRN_1423 | Positive | 34.37 |
| TP IRN_1424 | Negative | |
| TP IRN_1425 | Positive | 34.31 |
| TP IRN_1426 | Negative | |
| TP IRN_1427 | Positive | 34.77 |
| TP IRN_1428 | Negative | |
| TP IRN_1429 | Negative | |
| TP IRN_1430 | Positive | 33.11 |
| TP IRN_1431 | Negative | |
| TP IRN_1432 | Positive | 34.08 |
| TP IRN_1433 | Positive | 34.07 |
| TP IRN_1434 | Negative | |
| TP IRN_1435 | Positive | 35.91 |
| TP IRN_1436 | Negative | |
| TP IRN_1437 | Positive | 36.53 |
| TP IRN_1438 | Negative | |
| TP IRN_1439 | Negative | |
| TP IRN_1440 | Negative | |
| TP IRN_1441 | Positive | 34.94 |
| TP IRN_1442 | Negative | |
| TP IRN_1443 | Negative | |
| TP IRN_1444 | Negative | |
| TP IRN_1445 | Negative | |
| TP IRN_1446 | Negative | |
| TP IRN_1447 | Negative | |
| TP IRN_1448 | Negative | |
| TP IRN_1449 | Positive | 34.84 |
| TP IRN_1450 | Positive | 37.86 |
| TP IRN_1451 | Positive | 35.42 |
| TP IRN_1452 | Positive | 34.71 |
| TP IRN_1453 | Negative | |
| TP IRN_1454 | Positive | 36.53 |
| TP IRN_1455 | Negative | |
| TP IRN_1456 | Negative | |
| TP IRN_1457 | Positive | 33.11 |
| TP IRN_1458 | Negative | |
| TP IRN_1459 | Negative | |
| TP IRN_1460 | Positive | 33.87 |
| TP IRN_1461 | Negative | |
| TP IRN_1462 | Negative | 33.42 |
| TP IRN_1463 | Negative | |

| | | |
|---|---|---|
| TP IRN_1464 | Negative | |
| TP IRN_1465 | Positive | 33.21 |
| TP IRN_1466 | Positive | 30.96 |
| TP IRN_1467 | Positive | 32.47 |
| TP IRN_1468 | Positive | 33.19 |
| TP IRN_1469 | Positive | 28.66 |
| TP IRN_1470 | Positive | 33.18 |
| TP IRN_1471 | Positive | 31.10 |
| TP IRN_1472 | Negative | |
| TP IRN_1473 | Positive | 30.41 |
| TP IRN_1474 | Positive | 32.11 |
| TP IRN_1475 | Positive | 31.87 |
| TP IRN_1476 | Positive | 31.76 |
| TP IRN_1477 | Positive | 32.46 |
| TP IRN_1478 | Negative | 34.33 |
| TP IRN_1479 | Negative | |
| TP IRN_1480 | Positive | 36.57 |
| TP IRN_1481 | Positive | 34.26 |
| TP IRN_1482 | Negative | |
| TP IRN_1483 | Positive | 33.67 |
| TP IRN_1484 | Positive | 33.37 |
| TP IRN_1485 | Positive | 33.75 |
| TP IRN_1486 | Negative | |
| TP IRN_1487 | Positive | 33.42 |
| TP IRN_1488 | Positive | 32.70 |
| TP IRN_1489 | Positive | 33.98 |
| TP IRN_1490 | Negative | |
| TP IRN_1491 | Negative | |
| TP IRN_1492 | Positive | 34.76 |
| TP IRN_1493 | Negative | |
| TP IRN_1494 | Negative | |
| TP IRN_1495 | Positive | 34.90 |
| TP IRN_1496 | Positive | 34.40 |
| TP IRN_1497 | Negative | |
| TP IRN_1498 | Positive | 33.68 |
| TP IRN_1499 | Negative | |
| TP IRN_1500 | Positive | 32.53 |
| TP IRN_1501 | Positive | 31.95 |
| TP IRN_1502 | Positive | 33.27 |
| TP IRN_1503 | Negative | |
| TP IRN_1504 | Negative | |
| TP IRN_1505 | Negative | |
| TP IRN_1506 | Positive | 32.50 |
| TP IRN_1507 | Positive | 31.87 |
| TP IRN_1508 | Positive | 31.94 |
| TP IRN_1509 | Positive | 33.32 |
| TP IRN_1510 | Negative | |
| TP IRN_1511 | Negative | |
| TP IRN_1512 | Negative | |

| | | |
|---|---|---|
| TP IRN_1513 | Positive | 34.89 |
| TP IRN_1514 | Positive | 32.63 |
| TP IRN_1515 | Negative | |
| TP IRN_1516 | Positive | 32.46 |
| TP IRN_1517 | Negative | |
| TP IRN_1518 | Positive | 34.79 |
| TP IRN_1519 | Positive | 32.19 |
| TP IRN_1520 | Negative | |
| TP IRN_1521 | Positive | 31.70 |
| TP IRN_1522 | Positive | 33.15 |
| TP IRN_1523 | Positive | 33.84 |
| TP IRN_1524 | Positive | 32.10 |
| TP IRN_1525 | Positive | 32.81 |
| TP IRN_1526 | Positive | 35.54 |
| TP IRN_1527 | Negative | |
| TP IRN_1528 | Positive | 32.96 |
| TP IRN_1529 | Negative | |
| TP IRN_1530 | Positive | 27.56 |
| TP IRN_1531 | Positive | 30.91 |
| TP IRN_1532 | Positive | 32.91 |
| TP IRN_1533 | Positive | 34.17 |
| TP IRN_1534 | Positive | 37.08 |
| TP IRN_1535 | Positive | 33.68 |
| TP IRN 1536 | Positive | 32.67 |
| TP IRN 1537 | Positive | 32.15 |
| TP IRN 1538 | Positive | 35.54 |
| TP IRN 1539 | Positive | 33.54 |
| TP IRN 1540 | Positive | 30.86 |
| TP IRN 1541 | Positive | 32.23 |
| TP IRN 1542 | Positive | 28.46 |
| TP IRN 1543 | Positive | 32.71 |
| TP IRN 1544 | Negative | |
| TP IRN 1545 | Negative | |
| TP IRN 1546 | Negative | |
| TP IRN 1547 | Negative | |
| TP IRN 1548 | Negative | |
| TP IRN 1549 | Negative | |
| TP IRN 1550 | Negative | |
| TP IRN 1551 | Positive | 35.92 |
| TP IRN 1552 | Negative | |
| TP IRN 1553 | Negative | |
| TP IRN 1554 | Negative | |
| TP IRN 1555 | Negative | |
| TP IRN 1556 | Negative | |
| TP IRN 1557 | Negative | |
| TP IRN 1558 | Negative | |
| TP IRN 1559 | Negative | |
| TP IRN 1560 | Negative | |
| TP IRN 1561 | Negative | |

| | | |
|---|---|---|
| TP IRN 1562 | Negative | |
| TP IRN 1563 | Negative | |
| TP IRN 1564 | Negative | |
| TP IRN 1565 | Negative | |
| TP IRN 1566 | Positive | 24.19 |
| TP IRN 1567 | Positive | 30.05 |
| TP IRN 1568 | Positive | 30.62 |
| TP IRN 1569 | Negative | |
| TP IRN 1570 | Negative | |
| TP IRN 1571 | Positive | 32.55 |
| TP IRN 1572 | Positive | 32.71 |
| TP IRN 1573 | Positive | 32.77 |
| TP IRN 1574 | Positive | 32.87 |
| TP IRN 1575 | Positive | 32.94 |
| TP IRN 1576 | Positive | 33.21 |
| TP IRN 1577 | Negative | |
| TP IRN 1578 | Negative | 33.41 |
| TP IRN 1579 | Positive | 33.70 |
| TP IRN 1580 | Negative | 33.73 |
| TP IRN 1581 | Positive | 33.87 |
| TP IRN 1582 | Positive | 33.89 |
| TP IRN 1583 | Negative | 33.98 |
| TP IRN 1584 | Negative | 34.13 |
| TP IRN 1585 | Positive | 34.16 |
| TP IRN 1586 | Positive | 34.22 |
| TP IRN 1587 | Positive | 34.25 |
| TP IRN 1588 | Negative | |
| TP IRN 1589 | Positive | 34.27 |
| TP IRN 1590 | Positive | 34.44 |
| TP IRN 1591 | Positive | 34.52 |
| TP IRN 1592 | Positive | 34.56 |
| TP IRN 1593 | Negative | |
| TP IRN 1594 | Negative | |
| TP IRN 1595 | Positive | 34.65 |
| TP IRN 1596 | Negative | |
| TP IRN 1597 | Negative | |
| TP IRN 1598 | Positive | 34.74 |
| TP IRN 1599 | Negative | |
| TP IRN 1600 | Negative | |
| TP IRN 1601 | Negative | |
| TP IRN 1602 | Positive | 34.83 |
| TP IRN 1603 | Negative | |
| TP IRN 1604 | Negative | |
| TP IRN 1605 | Positive | 34.86 |
| TP IRN 1606 | Negative | |
| TP IRN 1607 | Negative | |
| TP IRN 1608 | Positive | 34.89 |
| TP IRN 1609 | Positive | 34.93 |
| TP IRN 1610 | Negative | |

| | | |
|---|---|---|
| TP IRN 1611 | Positive | 35.09 |
| TP IRN 1612 | Positive | 35.12 |
| TP IRN 1613 | Negative | |
| TP IRN 1614 | Negative | |
| TP IRN 1615 | Negative | |
| TP IRN 1616 | Negative | |
| TP IRN 1617 | Negative | |
| TP IRN 1618 | Positive | 35.21 |
| TP IRN 1619 | Negative | |
| TP IRN 1620 | Negative | |
| TP IRN 1621 | Positive | 35.35 |
| TP IRN 1622 | Negative | |
| TP IRN 1623 | Negative | |
| TP IRN 1624 | Positive | 35.37 |
| TP IRN 1625 | Negative | |
| TP IRN 1626 | Negative | |
| TP IRN 1627 | Negative | |
| TP IRN 1628 | Negative | |
| TP IRN 1629 | Negative | |
| TP IRN 1630 | Negative | |
| TP IRN 1631 | Negative | |
| TP IRN 1632 | Negative | |
| TP IRN 1633 | Negative | |
| TP IRN 1634 | Positive | 35.53 |
| TP IRN 1635 | Negative | |
| TP IRN 1636 | Negative | |
| TP IRN 1637 | Negative | |
| TP IRN 1638 | Negative | |
| TP IRN 1639 | Positive | 35.69 |
| TP IRN 1640 | Positive | 35.73 |
| TP IRN 1641 | Negative | |
| TP IRN 1642 | Negative | |
| TP IRN 1643 | Negative | |
| TP IRN 1644 | Negative | |
| TP IRN 1645 | Negative | |
| TP IRN 1646 | Negative | |
| TP IRN 1647 | Negative | |
| TP IRN 1648 | Positive | 35.79 |
| TP IRN 1649 | Negative | |
| TP IRN 1650 | Negative | |
| TP IRN 1651 | Negative | |
| TP IRN 1652 | Negative | |
| TP IRN 1653 | Negative | |
| TP IRN 1654 | Negative | |
| TP IRN 1655 | Negative | |
| TP IRN 1656 | Positive | 35.95 |
| TP IRN 1657 | Negative | |
| TP IRN 1658 | Positive | 35.99 |
| TP IRN 1659 | Negative | |

| | | |
|---|---|---|
| TP IRN 1660 | Positive | 36.03 |
| TP IRN 1661 | Negative | |
| TP IRN 1662 | Negative | |
| TP IRN 1663 | Negative | |
| TP IRN 1664 | Negative | |
| TP IRN 1665 | Negative | |
| TP IRN 1666 | Negative | |
| TP IRN 1667 | Negative | |
| TP IRN 1668 | Negative | |
| TP IRN 1669 | Positive | 36.23 |
| TP IRN 1670 | Negative | |
| TP IRN 1671 | Positive | 36.25 |
| TP IRN 1672 | Positive | 36.27 |
| TP IRN 1673 | Negative | |
| TP IRN 1674 | Negative | |
| TP IRN 1675 | Negative | |
| TP IRN 1676 | Positive | 36.38 |
| TP IRN 1677 | Negative | |
| TP IRN 1678 | Negative | |
| TP IRN 1679 | Positive | 36.49 |
| TP IRN 1680 | Negative | |
| TP IRN 1681 | Negative | |
| TP IRN 1682 | Negative | |
| TP IRN 1683 | Negative | |
| TP IRN 1684 | Positive | 36.64 |
| TP IRN 1685 | Negative | |
| TP IRN 1686 | Negative | |
| TP IRN 1687 | Negative | |
| TP IRN 1688 | Negative | 34.85 |
| TP IRN 1689 | Positive | 36.84 |
| TP IRN 1690 | Negative | |
| TP IRN 1691 | Positive | 36.89 |
| TP IRN 1692 | Negative | |
| TP IRN 1693 | Negative | |
| TP IRN 1694 | Negative | |
| TP IRN 1695 | Negative | |
| TP IRN 1696 | Negative | |
| TP IRN 1697 | Negative | |
| TP IRN 1698 | Negative | |
| TP IRN 1699 | Positive | 37.02 |
| TP IRN 1700 | Negative | |
| TP IRN 1701 | Positive | 37.06 |
| TP IRN 1702 | Negative | |
| TP IRN 1703 | Negative | |
| TP IRN 1704 | Positive | 37.07 |
| TP IRN 1705 | Negative | 37.09 |
| TP IRN 1706 | Positive | 37.10 |
| TP IRN 1707 | Negative | |
| TP IRN 1708 | Negative | |

| | | |
|---|---|---|
| TP IRN 1709 | Positive | 37.19 |
| TP IRN 1710 | Positive | 37.22 |
| TP IRN 1711 | Negative | |
| TP IRN 1712 | Negative | |
| TP IRN 1713 | Positive | 37.28 |
| TP IRN 1714 | Negative | |
| TP IRN 1715 | Positive | 37.33 |
| TP IRN 1716 | Negative | |
| TP IRN 1717 | Negative | |
| TP IRN 1718 | Negative | |
| TP IRN 1719 | Negative | |
| TP IRN 1720 | Negative | |
| TP IRN 1721 | Positive | 37.73 |
| TP IRN 1722 | Positive | 37.86 |
| TP IRN 1723 | Negative | |
| TP IRN 1724 | Negative | |
| TP IRN 1725 | Negative | |
| TP IRN 1726 | Negative | |
| TP IRN 1727 | Negative | |
| TP IRN 1728 | Negative | |
| TP IRN 1729 | Negative | |
| TP IRN 1730 | Negative | |
| TP IRN 1731 | Negative | |
| TP IRN 1732 | Negative | |
| TP IRN 1733 | Negative | |
| TP IRN 1734 | Positive | 31.89 |
| TP IRN 1735 | Negative | |
| TP IRN 1736 | Negative | |
| TP IRN 1737 | Positive | 33.35 |
| TP IRN 1738 | Negative | |
| TP IRN 1739 | Negative | |
| TP IRN 1740 | Negative | |
| TP IRN 1741 | Negative | |
| TP IRN 1742 | Positive | 35.15 |
| TP IRN 1743 | Negative | |
| TP IRN 1744 | Positive | 35.16 |
| TP IRN 1745 | Negative | |
| TP IRN 1746 | Negative | |
| TP IRN 1747 | Negative | |
| TP IRN 1748 | Negative | |
| TP IRN 1749 | Negative | |
| TP IRN 1750 | Negative | |
| TP IRN 1751 | Negative | |
| TP IRN 1752 | Negative | |
| TP IRN 1753 | Positive | 35.17 |
| TP IRN 1754 | Negative | |
| TP IRN 1755 | Negative | |
| TP IRN 1756 | Positive | 35.33 |
| TP IRN 1757 | Negative | |

| | | |
|---|---|---|
| TP IRN 1758 | Negative | |
| TP IRN 1759 | Positive | 35.34 |
| TP IRN 1760 | Negative | |
| TP IRN 1761 | Positive | 35.36 |
| TP IRN 1762 | Positive | 35.37 |
| TP IRN 1763 | Negative | |
| TP IRN 1764 | Negative | |
| TP IRN 1765 | Positive | 35.38 |
| TP IRN 1766 | Positive | 35.42 |
| TP IRN 1767 | Negative | |
| TP IRN 1768 | Positive | 35.47 |
| TP IRN 1769 | Positive | 35.49 |
| TP IRN 1770 | Negative | |
| TP IRN 1771 | Positive | 35.50 |
| TP IRN 1772 | Positive | 35.51 |
| TP IRN 1773 | Negative | |
| TP IRN 1774 | Negative | |
| TP IRN 1775 | Negative | |
| TP IRN 1776 | Positive | 35.52 |
| TP IRN 1777 | Negative | |
| TP IRN 1778 | Positive | 35.52 |
| TP IRN 1779 | Negative | |
| TP IRN 1780 | Positive | 35.52 |
| TP IRN 1781 | Negative | |
| TP IRN 1782 | Negative | |
| TP IRN 1783 | Negative | |
| TP IRN 1784 | Negative | |
| TP IRN 1785 | Negative | |
| TP IRN 1786 | Negative | |
| TP IRN 1787 | Negative | |
| TP IRN 1788 | Negative | |
| TP IRN 1789 | Negative | |
| TP IRN 1790 | Negative | |
| TP IRN 1791 | Negative | |
| TP IRN 1792 | Positive | 35.14 |
| TP IRN 1793 | Negative | |
| TP IRN 1794 | Negative | |
| TP IRN_1795 | Negative | |
| TP IRN_1796 | Negative | |
| TP IRN_1797 | Positive | 35.13 |
| TP IRN_1798 | Negative | |
| TP IRN_1799 | Negative | |
| TP IRN_1800 | Negative | |
| TP IRN_1801 | Negative | |
| TP IRN_1802 | Positive | 34.97 |
| TP IRN_1803 | Negative | |
| TP IRN_1804 | Negative | |
| TP IRN_1805 | Negative | |
| TP IRN_1806 | Negative | |

| | | |
|---|---|---|
| TP IRN_1807 | Positive | 34.89 |
| TP IRN_1808 | Negative | |
| TP IRN_1809 | Negative | |
| TP IRN_1810 | Negative | |
| TP IRN_1811 | Negative | |
| TP IRN_1812 | Positive | 34.86 |
| TP IRN_1813 | Negative | |
| TP IRN_1814 | Negative | |
| TP IRN_1815 | Negative | |
| TP IRN_1816 | Positive | 34.85 |
| TP IRN_1817 | Negative | |
| TP IRN_1818 | Negative | |
| TP IRN_1819 | Negative | |
| TP IRN_1820 | Negative | |
| TP IRN_1821 | Negative | |
| TP IRN_1822 | Negative | |
| TP IRN_1823 | Positive | 31.23 |
| TP IRN_1824 | Positive | 34.26 |
| TP IRN_1825 | Negative | |
| TP IRN_1826 | Negative | |
| TP IRN_1827 | Negative | |
| TP IRN_1828 | Negative | |
| TP IRN_1829 | Negative | |
| TP IRN_1830 | Negative | |
| TP IRN_1831 | Negative | |
| TP IRN_1832 | Negative | |
| TP IRN_1833 | Negative | |
| TP IRN_1834 | Negative | |
| TP IRN_1835 | Negative | |
| TP IRN_1836 | Negative | |
| TP IRN_1837 | Negative | |
| TP IRN_1838 | Negative | |
| TP IRN_1839 | Negative | |
| TP IRN_1840 | Negative | |
| TP IRN_1841 | Positive | 34.56 |
| TP IRN_1842 | Negative | |
| TP IRN_1843 | Negative | |
| TP IRN_1844 | Negative | |
| TP IRN_1845 | Positive | 34.59 |
| TP IRN_1846 | Negative | |
| TP IRN_1847 | Negative | |
| TP IRN_1848 | Negative | |
| TP IRN_1849 | Negative | |
| TP IRN_1850 | Positive | 34.73 |
| TP IRN_1851 | Positive | 34.74 |
| TP IRN_1852 | Negative | |
| TP IRN_1853 | Negative | |
| TP IRN_1854 | Negative | |
| TP IRN_1855 | Negative | |

| | | |
|---|---|---|
| TP IRN_1856 | Negative | |
| TP IRN_1857 | Negative | |
| TP IRN_1858 | Negative | |
| TP IRN_1859 | Negative | |
| TP IRN_1860 | Positive | 34.77 |
| TP IRN_1861 | Negative | |
| TP IRN_1862 | Negative | |
| TP IRN_1863 | Negative | |
| TP IRN_1864 | Negative | |
| TP IRN_1865 | Negative | |
| TP IRN_1866 | Negative | |
| TP IRN_1867 | Positive | 34.79 |
| TP IRN_1868 | Negative | |
| TP IRN_1869 | Positive | 34.82 |
| TP IRN_1870 | Negative | |
| TP IRN_1871 | Negative | |
| TP IRN_1872 | Negative | |
| TP IRN_1873 | Negative | |
| TP IRN_1874 | Negative | |
| TP IRN_1875 | Negative | |
| TP IRN_1876 | Positive | |
| TP IRN_1877 | Negative | 35.55 |
| TP IRN_1878 | Negative | |
| TP IRN_1879 | Negative | |
| TP IRN_1880 | Negative | |
| TP IRN_1881 | Negative | |
| TP IRN_1882 | Positive | 35.56 |
| TP IRN_1883 | Negative | |
| TP IRN_1884 | Positive | 35.59 |
| TP IRN_1885 | Negative | |
| TP IRN_1886 | Negative | |
| TP IRN_1887 | Negative | |
| TP IRN_1888 | Negative | |
| TP IRN_1889 | Negative | |
| TP IRN_1890 | Negative | |
| TP IRN_1891 | Negative | |
| TP IRN_1892 | Negative | |
| TP IRN_1893 | Negative | |
| TP IRN_1894 | Negative | |
| TP IRN_1895 | Negative | |
| TP IRN_1896 | Negative | |
| TP IRN_1897 | Negative | |
| TP IRN_1898 | Negative | |
| TP IRN_1899 | Negative | |
| TP IRN_1900 | Positive | 35.68 |
| TP IRN_1901 | Negative | |
| TP IRN_1902 | Negative | |
| TP IRN_1903 | Negative | |
| TP IRN_1904 | Positive | 35.73 |

| | | |
|---|---|---|
| TP IRN_1905 | Negative | |
| TP IRN_1906 | Negative | |
| TP IRN_1907 | Negative | |
| TP IRN_1908 | Negative | |
| TP IRN_1909 | Negative | |
| TP IRN_1910 | Negative | |
| TP IRN_1911 | Positive | 35.74 |
| TP IRN_1912 | Negative | |
| TP IRN_1913 | Negative | |
| TP IRN_1914 | Negative | |
| TP IRN_1915 | Positive | 35.74 |
| TP IRN_1916 | Negative | |
| TP IRN_1917 | Positive | 35.75 |
| TP IRN_1918 | Negative | |
| TP IRN_1919 | Negative | |
| TP IRN_1920 | Negative | |
| TP IRN_1921 | Negative | |
| TP IRN_1922 | Negative | |
| TP IRN_1923 | Negative | |
| TP IRN_1924 | Negative | |
| TP IRN_1925 | Negative | |
| TP IRN_1926 | Negative | |
| TP IRN_1927 | Negative | |
| TP IRN_1928 | Positive | 35.77 |
| TP IRN_1929 | Negative | |
| TP IRN_1930 | Negative | |
| TP IRN_1931 | Negative | |
| TP IRN_1932 | Negative | |
| TP IRN_1933 | Negative | |
| TP IRN_1934 | Negative | |
| TP IRN_1935 | Positive | 35.78 |
| TP IRN_1936 | Negative | |
| TP IRN_1937 | Negative | |
| TP IRN_1938 | Negative | |
| TP IRN_1939 | Negative | |
| TP IRN_1940 | Negative | |
| TP IRN_1941 | Negative | |
| TP IRN_1942 | Positive | 35.78 |
| TP IRN_1943 | Negative | |
| TP IRN_1944 | Negative | |
| TP IRN_1945 | Negative | |
| TP IRN_1946 | Negative | |
| TP IRN_1947 | Negative | |
| TP IRN_1948 | Negative | |
| TP IRN_1949 | Positive | 35.80 |
| TP IRN_1950 | Negative | |
| TP IRN_1951 | Negative | |
| TP IRN_1952 | Negative | |
| TP IRN_1953 | Negative | |

| | | |
|---|---|---|
| TP IRN_1954 | Negative | |
| TP IRN_1955 | Negative | |
| TP IRN_1956 | Negative | |
| TP IRN_1957 | Negative | |
| TP IRN_1958 | Negative | |
| TP IRN_1959 | Positive | 35.83 |
| TP IRN_1960 | Negative | |
| TP IRN_1961 | Negative | |
| TP IRN_1962 | Negative | |
| TP IRN_1963 | Positive | 35.86 |
| TP IRN_1964 | Positive | 36.05 |
| TP IRN_1965 | Negative | |
| TP IRN_1966 | Negative | |
| TP IRN_1967 | Negative | |
| TP IRN_1968 | Negative | |
| TP IRN_1969 | Negative | |
| TP IRN_1970 | Negative | |
| TP IRN_1971 | Negative | |
| TP IRN_1972 | Negative | |
| TP IRN_1973 | Negative | |
| TP IRN_1974 | Negative | |
| TP IRN_1975 | Negative | |
| TP IRN_1976 | Negative | |
| TP IRN_1977 | Negative | |
| TP IRN_1978 | Negative | |
| TP IRN_1979 | Negative | |
| TP IRN_1980 | Negative | |
| TP IRN_1981 | Negative | |
| TP IRN_1982 | Negative | |
| TP IRN_1983 | Negative | |
| TP IRN_1984 | Negative | |
| TP IRN_1985 | Negative | |
| TP IRN_1986 | Negative | |
| TP IRN_1987 | Negative | |
| TP IRN_1988 | Negative | |
| TP IRN_1989 | Negative | |
| TP IRN_1990 | Positive | 35.87 |
| TP IRN_1991 | Negative | |
| TP IRN_1992 | Negative | |
| TP IRN_1993 | Negative | |
| TP IRN_1994 | Negative | |
| TP IRN_1995 | Negative | |
| TP IRN_1996 | Positive | 35.90 |
| TP IRN_1997 | Negative | |
| TP IRN_1998 | Positive | 35.89 |
| TP IRN_1999 | Negative | |
| TP IRN_2000 | Negative | |
| TP IRN_2001 | Negative | |
| TP IRN_2002 | Negative | |

| | | |
|---|---|---|
| TP IRN_2003 | Negative | |
| TP IRN_2004 | Negative | |
| TP IRN_2005 | Positive | 36.03 |
| TP IRN_2006 | Negative | |
| TP IRN_2007 | Negative | |
| TP IRN_2008 | Positive | |
| TP IRN_2009 | Negative | |
| TP IRN_2010 | Negative | |
| TP IRN_2011 | Positive | 35.89 |
| TP IRN_2012 | Negative | |
| TP IRN_2013 | Negative | |
| TP IRN_2014 | Negative | |
| TP IRN_2015 | Negative | |
| TP IRN_2016 | Negative | |
| TP IRN_2017 | Negative | |
| TP IRN_2018 | Negative | |
| TP IRN_2019 | Negative | |
| TP IRN_2020 | Negative | |
| TP IRN_2021 | Positive | 35.95 |
| TP IRN_2022 | Negative | |
| TP IRN_2023 | Negative | |
| TP IRN_2024 | Positive | 36.07 |
| TP IRN_2025 | Positive | 36.09 |
| TP IRN_2026 | Negative | |
| TP IRN_2027 | Negative | |
| TP IRN_2028 | Negative | |
| TP IRN_2029 | Negative | |
| TP IRN_2030 | Negative | |
| TP IRN_2031 | Positive | 36.12 |
| TP IRN_2032 | Negative | |
| TP IRN_2033 | Negative | |
| TP IRN_2034 | Positive | 36.14 |
| TP IRN_2035 | Negative | |
| TP IRN_2036 | Negative | |
| TP IRN_2037 | Negative | |
| TP IRN_2038 | Negative | |
| TP IRN_2039 | Positive | 36.19 |
| TP IRN_2040 | Positive | 36.20 |
| TP IRN_2041 | Negative | |
| TP IRN_2042 | Negative | |
| TP IRN_2043 | Negative | |
| TP IRN_2044 | Negative | |
| TP IRN_2045 | Negative | |
| TP IRN_2046 | Positive | 36.21 |
| TP IRN_2047 | Negative | |
| TP IRN_2048 | Negative | |
| TP IRN_2049 | Negative | |
| TP IRN_2050 | Negative | |
| TP IRN_2051 | Positive | 36.22 |

| | | |
|---|---|---|
| TP IRN_2052 | Positive | 36.25 |
| TP IRN_2053 | Negative | |
| TP IRN_2054 | Negative | |
| TP IRN_2055 | Negative | |
| TP IRN_2056 | Negative | |
| TP IRN_2057 | Positive | 36.31 |
| TP IRN_2058 | Negative | |
| TP IRN_2059 | positive | 36.32 |
| TP IRN_2060 | Negative | |
| TP IRN_2061 | Negative | |
| TP IRN_2062 | Positive | 36.36 |
| TP IRN_2063 | Negative | |
| TP IRN_2064 | Negative | |
| TP IRN_2065 | Positive | 36.40 |
| TP IRN_2066 | Positive | 36.44 |
| TP IRN_2067 | Positive | 36.49 |
| TP IRN_2068 | Negative | |
| TP IRN_2069 | Positive | 36.49 |
| TP IRN_2070 | Positive | 36.53 |
| TP IRN_2071 | Negative | |
| TP IRN_2072 | Positive | 36.64 |
| TP IRN_2073 | Positive | 36.71 |
| TP IRN_2074 | Negative | |
| TP IRN_2075 | Positive | 36.80 |
| TP IRN_2076 | Positive | 36.81 |
| TP IRN_2077 | Positive | 36.85 |
| TP IRN_2078 | Positive | 36.90 |
| TP IRN_2079 | Negative | |
| TP IRN_2080 | Positive | 36.91 |
| TP IRN_2081 | Negative | |
| TP IRN_2082 | Negative | |
| TP IRN_2083 | Negative | |
| TP IRN_2084 | Positive | 36.95 |
| TP IRN_2085 | Positive | 36.96 |
| TP IRN_2086 | Negative | |
| TP IRN_2087 | Positive | 36.96 |
| TP IRN_2088 | Negative | |
| TP IRN_2089 | Positive | 36.96 |
| TP IRN_2090 | Positive | 37.01 |
| TP IRN_2091 | Positive | 37.03 |
| TP IRN_2092 | Positive | 37.06 |
| TP IRN_2093 | Negative | |
| TP IRN_2094 | Negative | |
| TP IRN_2095 | Positive | 37.07 |
| TP IRN_2096 | Positive | 37.12 |
| TP IRN_2097 | Positive | 37.15 |
| TP IRN_2098 | Positive | 37.24 |
| TP IRN_2099 | Negative | |
| TP IRN_2100 | Positive | 37.25 |

| | | |
|---|---|---|
| TP IRN_2101 | Positive | 37.33 |
| TP IRN_2102 | Positive | 37.39 |
| TP IRN_2103 | Positive | 37.46 |
| TP IRN_2104 | Positive | 37.59 |
| TP IRN_2105 | Positive | 37.65 |
| TP IRN_2106 | Negative | |
| TP IRN_2107 | Negative | |
| TP IRN_2108 | Positive | 37.69 |
| TP IRN_2109 | Negative | |
| TP IRN_2110 | Positive | 38.06 |
| TP IRN_2111 | Negative | |
| TP IRN_2112 | Positive | 39.55 |
| TP IRN_2113 | Negative | |
| TP IRN_2114 | Negative | |
| TP IRN_2115 | Positive | 37.90 |
| TP IRN_2116 | Negative | |
| TP IRN_2117 | Negative | |
| TP IRN_2118 | Positive | 38.07 |
| TP IRN_2119 | Negative | |
| TP IRN_2120 | Negative | |
| TP IRN_2121 | Positive | |
| TP IRN_2122 | Negative | |
| TP IRN_2123 | Negative | |
| TP IRN_2124 | Negative | |
| TP IRN_2125 | Negative | |
| TP IRN_2126 | Negative | |
| TP IRN_2127 | Negative | |
| TP IRN_2128 | Negative | |
| TP IRN_2129 | Negative | |
| TP IRN_2130 | Positive | 38.00 |
| TP IRN_2131 | Negative | |
| TP IRN_2132 | Negative | |
| TP IRN_2133 | Positive | 37.80 |
| TP IRN_2134 | Positive | 33.10 |
| TP IRN_2135 | Negative | |
| TP IRN_2136 | Positive | 34.10 |
| TP IRN_2137 | Negative | |
| TP IRN_2138 | Negative | |
| TP IRN_2139 | Negative | |
| TP IRN_2140 | Positive | 32.87 |
| TP IRN_2141 | Negative | |
| TP IRN_2142 | Negative | |
| TP IRN_2143 | Negative | |
| TP IRN_2144 | Positive | 33.80 |

## Appendix B : Research Ethics Approval

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

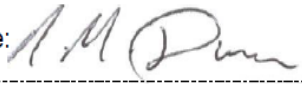
Faculty of Veterinary Science

**Research Ethics Committee**

| | |
|---|---|
| Project Title | **Improving the identification of Influenza A viruses in South Africa** |
| Project Number | **REC032-19** |
| Researcher / Principal Investigator | **Ms TP Phiri** |

| | |
|---|---|
| Dlissertation / Thesis submitted for | **Masters** |

| | |
|---|---|
| Supervisor | **Prof C Abolnik** |

| | |
|---|---|
| **APPROVED** | Date: **2019-10-11** |
| CHAIRMAN: UP Research Ethics Committee | Signature:  |

# Appendix C : Section 20 Ethical Approval

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
**REPUBLIC OF SOUTH AFRICA**

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

**Enquiries:** Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
**Reference:** 12/11/1/1/8 (1217)

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SARChI Chair in Poultry Health and Production
University of Pretoria
Tel: +27 (0)12 529 8258
E-mail: Celia.abolnik@up.ac.za

Dear Prof Abolnik

**RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984)**

Your application dated 2 July 2019 requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers. I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

**Conditions:**

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. This permission is given upon finding the biosecurity of the research project as described to be acceptable to DAFF. This permission does not serve as any approval or endorsement by DAFF for the validation, commercial use or registration of any influenza test for any purpose in South Africa. Please apply to the Director: Animal Health for more information regarding validation of tests for controlled animal diseases;
3. The research project is approved as per the application form dated 2 July 2019 and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this research project under this Section 20 permit. Please apply in writing to HerryG@daff.gov.za;

4. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 permit. Please apply in writing to HerryG@daff.gov.za;
5. No live animals may be used in this study under this Section 20 permit;
6. All work with live influenza viruses must be performed in the DAFF approved Poultry Research Unit BSL 3 Facility (DAFF-C02) at the Faculty of Veterinary Science, Onderstepoort;
7. All samples or material potentially containing live influenza virus must be inactivated with TRIzol® LS Reagent before they may be released from the Poultry Research Unit BSL 3 Laboratory (DAFF-C02);
8. Only South African strains of avian influenza viruses may be used in this research project. South African strains of avian influenza virus required that are not stored at the Poultry Research Unit may be requested from ARC-OVR;
9. All potentially infectious material utilised or generated during or by the research project is to be destroyed at completion of the research project. Only a registered waste disposal company may be used for the removal of all potentially infectious waste from the research project;
10. Records must be kept for five years for auditing purposes;
11. A dispensation for the storage of bacterial plasmids containing cloned HA and NA genes is attached;

**Title of research/study:** Improving the identification of Influenza A viruses detected in wild birds and ostriches
**Researcher:** Prof Celia Abolnik
**Institution:** The Poultry Research Unit BSL 3 facility (DAFF-C02), the Poultry Research Unit Broiler Room 3 and the Poultry Research Laboratory (BSL2) at the Faculty of Veterinary Science, Onderstepoort;
**Permit Expiry date:** 30 July 2021
**Our ref Number:** 12/11/1/1/8 (1217)
**Your ref**: REC032-19

Kind regards,

**DR. MPHO MAJA**
**DIRECTOR OF ANIMAL HEALTH**
**Date:** 2019 -07- 10



*SUBJECT:* *PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)*

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
**REPUBLIC OF SOUTH AFRICA**

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

**Enquiries:** Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
**Reference:** 12/11/1/1/8 (1217)

Professor Celia Abolnik
SARChI Chair in Poultry Health and Production
University of Pretoria
Tel: +27 (0)12 529 8258
E-mail: Celia.abolnik@up.ac.za

**RE: DISPENSATION ON SECTION 20 APPROVAL IN TERMS OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984) FOR: "IMPROVING THE IDENTIFICATION OF INFLUENZA A VIRUSES DETECTED IN WILD BIRDS AND OSTRICHES"**

A dispensation is hereby granted on Point 9 of the Section 20 approval that was issued for the above mentioned study (attached):

i) Bacterial plasmids containing cloned HA and NA genes must be stored under access control at the DAFF approved Poultry Research Unit BSL 2 facility;

ii) Bacterial plasmids containing cloned HA and NA genes must not be outsourced or used for further research without prior written approval from the Director: Animal Health.

Kind regards,

**DR. MPHO MAJA**
**DIRECTOR: ANIMAL HEALTH**
**Date:** 2019 -07- 10