Avian Influenza

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Summary

Avian influenza (AI) is caused by viruses that are members of the *Influenza A* genus of the family *Orthomyxoviridae*. Of the three influenza genera (A, B and C), only influenza A viruses are known to infect birds and these are further classified on the basis of their haemagglutinin (H) and neuraminidase (N) surface antigens. Highly pathogenic avian influenza (HPAI) (once known as fowl plague) is caused by specific type A influenza viruses. Because infection with type A influenza viruses can be associated with a wide range of clinical signs, diagnosis depends on identification and characterisation of the virus. As HPAI refers to infection with virulent strains of influenza A virus, it is necessary to assess the virulence of an isolate for domestic poultry. All HPAI strains isolated to date have been either of the H5 or H7 subtype. However, there are many H5 or H7 isolates that have been of low virulence. Low pathogenicity H5 and H7 subtypes are not endemic in Australian and New Zealand commercial poultry flocks, however there have been occasional detections in commercial duck flocks in Australia.

Clinical signs may vary according to the strain of virus, host, concurrent and secondary infections with other organisms and environmental conditions. Infection with avian influenza viruses may be inapparent or range from respiratory, enteric and reproductive disease to a peracute systemic infection with a 100% mortality rate. Disease caused by HPAI viruses may vary from one of sudden death with few or no overt signs to a more characteristic disease with respiratory signs, excessive lacrimation, sinusitis, oedema of the head, cyanosis of the unfeathered skin, nervous signs and diarrhoea. However, none of these signs can be considered pathognomonic.

Since 2002 isolates of H5N1 viruses from Asia, Europe and Africa have caused disease and deaths in many species of wild aquatic birds, including ducks and geese, whereas earlier isolates affected mainly chickens and turkeys. Some H5N1 HPAI strains have also caused disease and deaths in humans, raising public and occupational health concerns. In some circumstances, virus isolation and in vivo assessments of virulence have been replaced by, or supplemented with, reverse-transcription polymerase chain reaction and nucleotide sequencing. There are well-recognised nucleotide sequences that are present in HPAI virus

strains allowing classification of viruses into high or low pathogenicity. Subtyping of virus isolates may be done using monospecific antiserum against each of the H and N proteins or by PCR but such typing is usually restricted to reference laboratories.

Serological testing of birds infected with influenza viruses can identify the responsible virus broadly as a Type A virus at a group level and also more specifically at the subtype level. As all influenza A viruses have antigenically similar nucleocapsid and matrix antigens, antibodies to these shared antigens can be detected in Type A group serological tests using agar gel immunodiffusion (AGID) tests and enzyme-linked immunosorbent assays (ELISA). However, not all species of birds develop demonstrable precipitating antibodies, limiting the use of the AGID. The ELISA has not been validated for use in all avian species but has been thoroughly evaluated for use in domestic chickens. Viruses are subtyped on the basis of the haemagglutinin (H) and neuraminidase (N) antigens. As the haemagglutinin antigen is subtype specific, haemagglutination inhibition (HI) tests are useful to identify the subtype(s) with which birds have been infected. HI tests have also been employed for routine Type A serology but, because of their subtype specificity, infections with other subtypes may be missed. Neuraminidase inhibition assays used to identify the N antigen subtype are more complex and not routinely employed for large scale serology.

This document describes those procedures that may be used for the detection of infection or diagnosis of disease in domestic poultry. Procedures that are used for determining the virulence of influenza viruses *in vivo* are not included as these activities are restricted to the Avian Influenza Reference Laboratory at the Australian Animal Health Laboratory (AAHL).

NOTE: In Australia it is the policy of Animal Health Committee and SCAHLS that the cultivation of or the inoculation of birds or animals with viruses from the H5 or H7 subtypes, or other isolates that may be classified as virulent, will be carried out only at the Australian Animal Health Laboratory (AAHL). **Due to the possibility of human infection and high risk to the poultry industry and the environment, handling of such agents while being prepared for transfer to AAHL should be conducted under a high level of biosecurity.** In New Zealand, propagation of HPAI (including subtype H5 and H7) will be carried out at MAF-AHL PC3+ laboratory.

Part 1. Diagnostic Overview

1. Aetiology

Avian influenza (AI) is caused by viruses that are members of the *Influenza A* genus of the family *Orthomyxoviridae*. Of the three influenza genera (A, B and C), only influenza A viruses are known to infect birds. They have a negative strand, segmented genome of eight segments coding for 10 proteins. Influenza A viruses share common epitopes on the nucleocapsid (NP) and matrix (M) proteins, but are further classified into subtypes based on the surface glycoproteins, the haemagglutinin (H) and neuraminidase (N).[1] At present, 16 haemagglutinin subtypes (H1–H16) and 9 neuraminidase subtypes (N1–N9) are recognised and any combination of H and N is possible. All subtypes of influenza A viruses have been isolated from wild aquatic birds but other species of birds and mammals may be infected.[1,2]

2. Assessment of pathogenicity and notifiable disease status of influenza viruses

Highly pathogenic avian influenza (HPAI) (once known as fowl plague) is caused by virulent H5 and H7 subtypes of A influenza viruses. The characterisation of a virus as highly pathogenic is based on *in vivo* virulence assessments in chickens or molecular analysis to identify a characteristic nucleic acid sequence coding for multiple basic amino acids at the cleavage site of the haemagglutinin precursor protein HA_0.[1,2,3] All highly virulent strains isolated from disease outbreaks to date have been either of the H5 or H7 subtype but other subtypes have been detected with virulent characteristics.[4] The detection of virus infection or the suspicion of infection, with any highly pathogenic or virulent influenza virus, must be notified to the relevant regulatory authority (ultimately the Chief Veterinary Officer) in accordance with national and international (OIE) regulations.[3,5] While there are many H5 or H7 isolates that have been of low virulence, because of the propensity for these to mutate rapidly to highly pathogenic strains during passage in domestic poultry, infections of domestic poultry with any H5 or H7 virus are notifiable internationally. In some Australian states, infection of poultry with any influenza virus is notifiable. Assessment of virulence by HA gene sequencing may be done in any suitable facility but the results must be confirmed by the Reference Laboratory. In vivo assessments of the virulence of influenza viruses must be done in an Influenza Reference Laboratory.

3. Clinical signs in birds

Avian influenza is a disease affecting mainly chickens and turkeys, but disease may occur in a wide variety of species, especially after infection with the H5N1 subtype.[2,6,7] Clinical signs may vary according to the strain of virus, host, secondary infections and environmental conditions.[8,9,10] Infection with avian influenza viruses (AIV) may be inapparent or range from respiratory, enteric and reproductive disease to a peracute systemic infection with a 100% mortality rate.[11,12,13]

Signs of infection with HPAI viruses may vary from sudden death with few or no overt signs to a more characteristic disease with respiratory signs, excessive lacrimation, sinusitis, oedema of the head, cyanosis of the unfeathered skin, nervous signs and diarrhoea.[2,4,6] However, none of these signs can be considered pathognomonic. Isolates of Asian lineage H5N1 HPAI viruses since 2002 have caused disease and deaths in many species of poultry and wild birds, including ducks, geese,[9,10,12,13], whereas earlier isolates affected mainly chickens and turkeys.[11,14]

All subtypes of AIV have been identified in anatid species, which are believed to be a major reservoir for influenza viruses.[8,15,16] However, with the exception of recent Asian lineage

H5N1 HPAI isolates, there has been little or no disease observed in waterfowl and other wild birds.[7,8,17,18,19]

4. Infections of other species

Human infections with AIV subtypes H3, H4, H5, H6, H7, H9 and H10 have been reported.[21,22,23,63] Some HPAI strains have caused disease and deaths in humans, raising public and occupational health concerns.[20,23] Various other mammals have been infected with influenza viruses of avian origin but most infections have been asymptomatic. Disease due to influenza virus infection has been observed in pigs, cats (both domestic and large feline species), dogs and horses, but disease due to notifiable subtypes of AI has only been reported in cats (domestic and large wild species) and dogs.[24,25,26]

5. Epidemiology

Avian influenza viruses are not very resistant to warm temperatures, but they remain viable for longer periods in a cool and humid environment.[1] Environmental conditions have a marked effect on virus survival outside the bird.[27,30] Survival in aerosols is prolonged by high relative humidity and low temperature [32], and survival in faeces is prolonged by high moisture levels and low temperature.[28,29] AIV can survive in faeces at 4°C for at least 35 days [29], and virus has survived in dust in poultry houses for two weeks after depopulation.[34] AIV is stable over a pH range of 5.5–8 but is rapidly inactivated by heat, disinfectants and detergents.[28,30,31,33]

Wild avian species, especially waterfowl, provide an important reservoir for AIV, which can be isolated from water sources where waterfowl are present.[2,10,27] Untreated or improperly treated water from dams or creeks that has been contaminated with waterfowl faeces has been implicated as the source of virus for many outbreaks, including several that have occurred in Australia.[36,37,38,39]

In some countries movements of live birds, including vaccinated birds, through markets have played an important role in the maintenance and dissemination of AIV.[10] Virus can spread very rapidly and can also be carried over long distances by transport of contaminated materials such as bird cages, pallets, egg filler flats, manure and feed, and on contaminated clothing, equipment and vehicles.[16] Contaminated products such as meat and eggs can spread virus but are less important than other mechanisms.[35] A more detailed description of various aspects of the epidemiology and other field aspects of AI can be found in the AUSVETPLAN manual.

6. Occurrence and distribution

From 1959 to 1990 there were ten reported outbreaks of HPAI worldwide. All of these occurred in temperate areas and, with one exception, were in chickens and turkeys.[1,7] Since 1990 there has been an expansion of the range of HPAI outbreaks and in particular the Asian continent with a large increase in the number of AIV outbreaks.[40,41] Australia has had five recorded outbreaks of HPAI between 1976 and 1997, all caused by H7 AI viruses.[37,38,39,42,43,44] All were confined to one, two or three premises and were controlled by eradication and quarantine. All AIV subtypes (H1- H16 and N1 – N9) have been identified in free-living birds in Australia (P Selleck and P Kirkland, unpublished).[45,46,47,61,62] There have been no outbreaks of HPAI in commercial poultry in New Zealand but recent surveys have detected notifiable subtypes of AIV in wild birds.[48]

7. Gross and microscopic pathology

The respiratory, enteric and reproductive tracts are the main body systems to show signs of infection with an AIV.[1,2,6] The lesions associated with infection with virulent AIV consist of a combination of haemorrhagic, congestive and transudative changes. In the early stages these may be present in the comb and wattles and be followed by necrotic changes in the liver, lungs, pancreas, spleen, kidneys and breast muscle.[4,6,8] Petechial and ecchymotic haemorrhages may be seen in the intestines and internal organs.[9,10] A greyish-yellow exudate may be present on the air-sacs and peritoneal surfaces and there may be fibrin deposits on the pericardium and liver. Sinusitis of varying intensity has been reported in outbreaks in a wide range of bird species, including game birds.[10,11,12,13,14]

In less severe infections, such as with mildly pathogenic AIV, lesions may be seen in the sinuses and respiratory tract, characterised by catarrhal, serofibrinous, mucopurulent or caseous inflammation.[8,14] Catarrhal to fibrinous peritonitis and egg yolk peritonitis may be seen. Catarrhal to fibrinous enteritis may be seen in the caeca and/or intestine, particularly in turkeys. Exudates may be seen in the oviducts of laying birds.[8,14]

The microscopic lesions seen in the gross changes described above are not definitive for HPAI, although vasculitis in the brain and other organs may be highly suggestive of the disease.[4,7,8,9,10,12,13,14]

8. Diagnostic tests and specimens

Specimens are likely to be examined for evidence of AIV infection for:
(a) the diagnosis of specific disease incidents;
(b) certification of freedom from infection or the health status of individual birds or flocks;

8.1 Disease diagnosis

For disease diagnosis, it is necessary to identify the subtype of AIV that has infected the diseased birds and to assess the virulence of the strain.[3] These objectives are achieved by virus isolation.[49] The isolate is then available for both *in vivo* studies of virulence and *in vitro* characterisation to determine the H and N subtype, as well as the pathogenicity by nucleic acid sequencing. Virus isolation using specimens of avian origin is usually done by inoculating embryonated chicken eggs.[3] After incubation for up to 5 days, virus replication is monitored by testing allantoic fluids for the presence of haemagglutinin. Any fluids that haemagglutinate erythrocytes are tested against specific antisera to demonstrate the presence of an AIV Type A and subsequently the H and N subtypes.[3,49]

Molecular assays, especially the polymerase chain reaction (PCR), allow rapid testing to be undertaken directly on specimens and can indicate or exclude the presence of AIV of interest. PCR assays that are type A pan-reactive or are either H5- or H7-specific are available[50,51,52]. Specimens in which RNA from an AIV Type A has been detected can be further tested in H5 and H7 specific assays.[57,58,59] In an emergency, both group and subtype-specific assays can be run concurrently. The detection of RNA from a H5 or H7 virus does not constitute a diagnosis of AI but would provide a very high level of suspicion while other investigations are continuing. These assays can potentially be completed within several hours and, in addition to speed, after initial specimen processing has been completed, allow a laboratory to test specimens that no longer contain live virus. They are also ideally suited to high through-put semi-automated testing. Consequently these are now the preferred assays for primary disease

exclusion testing and large scale screening that may be undertaken during surveillance testing associated with a disease outbreak or other large scale AI surveys.

Nucleic acid sequencing can also be undertaken without culturing of the virus but can be time-consuming and will be limited by the quantity and quality of RNA in the specimen. When a virus isolate is available, nucleic acid sequencing can be undertaken more efficiently and critical segments of the genome can be investigated in detail.

A range of rapid diagnostic tests that are designed to detect viral antigens are available from commercial sources. These 'point of care' (POC) or 'pen-side' tests do not have the sensitivity of virus isolation or PCR but are usually able to detect viral antigens in birds that are clinically affected with HPAI. There are a range of different test formats, usually in a 'dip-stick' or chromatographic strip style, some of which are Type A reactive and others that are H5 specific. They should be regarded as 'flock' tests and while a negative result does not exclude AI, a positive result can be highly suggestive of infection with an avian influenza virus.[53] It should be noted that the use of these devices is subject to regulatory control in some Australian states.

The types of serological tests commonly used for testing for AI antibody are:

(a) The Agar Gel Immunodiffusion (AGID) test;
(b) The Haemagglutination Inhibition (HI) test;
(c) The antibody ELISA.

Each of these tests has a role in the diagnosis of AI. The choice of one or more of these tests depends to a large degree on the purpose for the testing. The AGID test is recognised to be group reactive and will reliably detect antibody to all subtypes of AIV in chickens and turkeys, however precipitating antibodies are not produced by all avian species.[54,55] Ducks and other wild birds may not produce detectable levels of precipitating antibodies and should not be tested in the AGID.[1,2,3,] In contrast, while the HI test will detect antibodies in all avian species, it is much less broadly reactive and reflects a relatively high degree of haemagglutinin subtype specificity. When used on a flock basis for commercial poultry, it will usually reliably identify the haemagglutinin subtype of the virus that has infected the birds.[3,4,49] The blocking ELISA (bELISA) will detect antibodies to any Type A AIV and is likely to detect antibodies in any avian species.[56] While the test has been fully validated for use in chickens, there is uncertainty of the threshold values for positive results in wild bird species. Nevertheless, it is believed that negative results in any bird species will indicate a lack of exposure to Type A AIV.[56] The bELISA has advantages of both very high sensitivity and specificity and is the test of choice for large scale testing. Several commercial ELISAs that are believed to be specific for the detection of antibodies to H5 and N1 viruses have recently become available but none has been evaluated for use in Australia or New Zealand.

8.2 Testing for certification of freedom from infection with AIV

Certification of freedom from infection with AIV is usually undertaken for one of two purposes:

a) Freedom from infection during surveillance after an outbreak, or
b) Health certification before movement between regions of differing AIV status or for export.

For surveillance after an outbreak, testing to demonstrate freedom from AIV infection is most efficiently and effectively undertaken by testing a statistically significant number of serum samples although qRT-PCR may be appropriate in some circumstances. The bELISA may be

used to test for antibodies to all AI viruses or the HI test may be used to test for antibodies specific for the outbreak virus. When testing is required for health certification, the test(s) selected will depend on the requirements of the regulatory authority. There may be a requirement to test for freedom from both virus and antibodies, especially with non-commercial poultry species.

8.3 Collection and storage of specimens

When samples are taken from diseased birds for diagnostic purposes, a wide range of specimens should be collected from at least 5 birds and should include swabs, fresh and formalin-fixed tissues, and sera. Virus detection, either by PCR or virus isolation, is undertaken on the swabs and fresh tissues.

Samples from live birds should include both tracheal and cloacal swabs as well as clotted blood. Oropharyngeal swabs are preferred over tracheal swabs when H5N1 infection is suspected. As small delicate birds may be harmed by swabbing, the collection of fresh faeces may serve as an adequate alternative. Faecal samples (approximately 1 g) may be submitted fresh or by collection on a liberally coated swab[3,4,49] that is subsequently placed in transport medium (see below).

Samples from dead birds should include cloacal and oropharyngeal or tracheal swabs. Samples of fresh and formalin-fixed trachea, lungs, air sacs, intestine, spleen, pancreas, kidney, brain, liver and heart should also be collected. Virus detection is undertaken on fresh tissues and swabs while histopathology is undertaken on the formalin-fixed tissues. Sections from the formalin-fixed tissues may be stained with standard histological stains or with specific antibodies for immunohistochemistry.

Swabs should be placed in a viral transport medium immediately after collection and kept chilled while being transported to the laboratory. Phosphate buffered gelatin saline (PBGS, pH 7.4) containing antibiotics is recommended, but other transport media are suitable provided they give suitable buffering capacity to control pH changes and have components to stabilise the virus. On receipt at the laboratory, the cotton-tipped swab should be removed from the PBGS and the fluid held at 4◦C. If testing cannot commence within 3-4 days, the specimens should be frozen at or below -70◦C. Freezing and thawing more than once will result in a significant reduction in the virus titre.

For large scale surveillance purposes in healthy flocks, only swabs and blood should be collected. Swabs can be processed rapidly in high throughput systems with little or no effect on sensitivity compared with examination of homogenised tissue samples.

Blood samples should be held at room temperature (about 20-25^{o}C) until the serum has separated then chilled during transport to the laboratory. The serum should be decanted from the clot within 24-48 hours of collection and can be stored for about 4-6 weeks at 4°C without significant decline in antibody titre. Freezing at -20°C or lower is preferred for longer storage but repeated freeze-thaw cycles should be avoided.

Type A Influenza Virus Infections

Part 2: Diagnostic Test Methods

1. Detection of Type A influenza viruses

For diagnostic and surveillance purposes, the objective is to identify the presence of a Type A influenza virus (or its nucleic acid or antigens) in the specimens that have been submitted to a laboratory. Each method has a role in influenza diagnosis but each method has limitations. Usually the most information can be obtained following virus isolation (culture) as the isolate is available for complete characterisation, nucleic acid sequencing, determination of pathotype and virulence as well as other *in vitro* and *in vivo* studies. However, virus isolation depends on the presence of viable virus in the specimen and availability of appropriate virus isolation capabilities. As virus isolation involves amplification of the virus, usually to high titre, investigation of disease incidents potentially caused by highly pathogenic viruses or surveillance testing in which H5 or H7 have been detected by PCR is restricted to AAHL. Virus isolation on surveillance samples may be done in other laboratories.

Virus isolation methods are also time consuming and take longer to obtain a result than most other procedures. Molecular methods such as PCR allow the detection of nucleic acid from non-infectious virus, whether the virus is no longer viable in the original specimen or has been rendered non-infectious during initial processing in the laboratory or its infectivity is reduced by the presence of antibodies in the sample. As well as being rapid, some PCR methods will identify the virus subtype, potentially virulent strains and are amenable to semi-automation, allowing high throughput. Limited nucleic acid sequencing can be undertaken on the PCR products to assist with virus identification. Rapid antigen detection kits for field or laboratory use have become available commercially, and, if used under controlled circumstances, may be of value during the investigation of a disease outbreak.

NOTE: In Australia it is the policy of Animal Health Committee and SCAHLS that the cultivation of, or inoculation of birds or animals with, viruses from the H5 or H7 subtypes, or other isolates that may be classified as virulent, will only be carried out at AAHL. Due to the possibility of human infection and high risk to the poultry industry and the environment, handling of such agents while being prepared for transfer to AAHL should be conducted under a high level of biosecurity (physical containment level 3 [PC3] wherever possible). In New Zealand, propagation of HPAI (including subtype H5 and H7) will be carried out at MAF-AHL PC3+ laboratory. Also, the diagnostic methods presented here will be conducted in New Zealand AHL with some modifications to reflect unique New Zealand AI status.

1.1 Specimen collection

Samples from dead birds should include tracheal or oro-pharyngeal swabs, cloacal swabs or faeces. A range of fresh and formalin-fixed tissues, including trachea, lung, spleen, pancreas, kidney, brain, liver and heart should also be collected. When investigating a disease event, it is preferable for fresh specimens (tissues and swabs) to be collected separately and a decision on whether to pool samples can be made at the laboratory. If specimens are pooled and sufficient

material is available, it is preferable to limit the range of organs included in a single pool and restrict pools to tissues from the same body system.

Samples from live birds should include both cloacal and tracheal swabs as different isolates may be preferentially excreted by different routes. Some Asian H5N1 viruses have respiratory tract tropisms, whereas Australian H7 viruses have been isolated most frequently from cloacal swabs. If samples are to be taken from small birds use paediatric swabs or collect faeces.

Swabs should be placed in a viral transport medium immediately after collection and kept chilled while being transported to the laboratory. Sterile containers should contain a maximum of 3 mL of transport medium. Transport medium may be phosphate buffered gelatin saline (PBGS, pH 7.4) (the recommended transport medium), cell culture medium, or brain heart infusion broth, each containing antibiotics (see section 1.2.1 below). Tissues should be individually collected into sterile containers without transport medium.

On receipt at the laboratory, it is preferable for the cotton-tipped swab to be removed from the transport medium and discarded, and the fluid held at 4°C. If testing cannot commence within 3-4 days, the specimens should be frozen at or below -70°C. Freezing and thawing more than once will result in a significant reduction in the virus titre but will have less impact on PCR results. DO NOT store specimens at -20^{o}C and avoid repeated freezing and thawing.

1.2 Specimen preparation

Faeces and tissues should be homogenised as 10–20% (w/v) suspensions in transport medium. Suspensions should be held for 1–2 h at room temperature to allow the antibiotics to take effect and then clarified by centrifugation at 2500 *g* for 5 min. Filtration to overcome bacterial contamination should be avoided but, if this is necessary, avoid the use a filter less than 0.45 um where possible. Centrifugation can also remove contaminating bacteria but this will also reduce the viral titre.

1.2.1 Reagents for specimen collection and preparation

Antibiotics – cell culture grade:

Penicillin (2000 units/mL)
Streptomycin (2 mg/mL)
Gentamycin (50 µg/mL)
Mycostatin (1000 units/mL)

Five-fold higher concentrations of antibiotics should be used for faeces and cloacal swabs.

Phosphate buffered saline (PBS)

Sodium chloride, NaCl	8.0 g
Potassium chloride, KCl	0.2 g
Disodium hydrogen phosphate, Na_2HPO_4	2.9 g
Potassium dihydrogen phosphate, KH_2PO_4	0.2 g

Make up to 1 L with water and adjust pH to 7.4 after adding the antibiotics.

Phosphate buffered gelatin saline (PBGS)

Phenol red	0.4 mg
Gelatin	5.0 g
PBS (pH 7.4)	1 L

Dissolve gelatin in PBS at 56◦C, add phenol red, autoclave at 121◦C for 20 min and dispense aseptically in 5 mL volumes.

1.3 Virus Isolation Procedures

The preferred method of isolation of avian influenza viruses is by inoculation into the allantoic sac of 9-11 day-old specific pathogen-free (SPF), embryonated chicken eggs. If SPF eggs are not available, eggs from antibody-free flocks may be used. A variety of cell cultures have been used to culture HPAI viruses but low pathogenic avian influenza (LPAI) viruses require the addition of trypsin to the medium to support replication of the virus. Growth of virus in cell cultures without trypsin may be used as a differential diagnosis of HPAI.

NOTE: If virus isolation is undertaken in a State veterinary laboratory, the specimens must be first screened by PCR and, if positive in a PCR assay for Type A influenza viruses, should be tested for H5 and H7 subtypes with negative results.

The supernatant fluids obtained after centrifugation at 2500 *g* are inoculated into the allantoic sac of 9-11 day old, embryonated SPF chicken eggs. A volume of 0.1 mL may be inoculated into five SPF eggs or, if eggs are in short supply, 0.2 mL into three SPF eggs. Eggs are incubated at 37°C for 5 days. Some highly pathogenic viruses may kill eggs within 24 hours so all eggs that die at any stage during the incubation are tested for the presence of haemagglutinating (HA) agents. HPAI viruses will usually kill inoculated eggs within 48 hours. Eggs alive after 5 days should be chilled at 4°C for a minimum of 4 h and the allantoic fluids then tested for HA agents. Detection of HA activity indicates a high probability of the presence of an influenza A virus or of an avian paramyxovirus. Fluids that give a negative reaction on first passage should be passaged into at least one further batch of eggs. Specimens from wild birds should be passaged a third time, especially if egg deaths, without haemagglutination, occur in the second passage.

1.4 Identification of the agent

Any haemagglutinating activity in an allantoic fluid should be identified, but because many viruses and bacteria cause haemagglutination, agents other than influenza viruses should be considered. The presence of an influenza virus in allantoic fluids may be confirmed by HI using mono-specific antisera. Several commercial ELISA kits are available for the detection of Type A influenza viruses, but these do not identify the H or N subtypes of the virus. Alternatively, the presence of an influenza virus can be confirmed by PCR using group A primers or H5 or H7 specific primers (see Section 1.5 below).

1.5 Detection of Type A Influenza viruses by polymerase chain reaction (PCR) assays

Reverse transcriptase polymerase chain reaction (RT-PCR) assays are now frequently used for the detection of nucleic acid from influenza viruses. Through the use of specific primers and probes, detailed information on the subtypes and pathogenicity of avian influenza viruses can be obtained. These assays are very sensitive and very quick and have the advantage of being able to detect nucleic acid from virus that is either no longer infectious or is defective. Buffers used during nucleic acid extraction procedures rapidly inactivate influenza viruses, thereby minimising biosecurity concerns during subsequent steps of the assay. However, extreme care

must be taken to ensure that there is not cross-contamination of materials or reagents or between different steps of the procedure as a result of the release of amplified nucleic acid into the environment. It is strongly recommended that the different steps in the assay are conducted in completely separate laboratories (in accordance with SCAHLS recommendations for PCR – Veterinary Laboratory Guidelines for Nucleic Acid Detection Techniques.[60] Staff involved in the procedure should move systematically between laboratories during a working day, starting in 'clean' areas (where there is no PCR product generated) and moving to areas that are progressively more likely to be contaminated with PCR product last. It is also essential that sufficient controls are included in the assay to monitor for contamination and false positive results. The use of 'real time' PCR assays minimises these problems.

1.5.1 'Real Time' polymerase chain reaction assays

'Real time' or quantitative (q) RT-PCR assays are now recommended for influenza virus detection for both diagnostic and surveillance purposes due to the very high sensitivity and specificity for both the detection of Type A influenza viruses at a group level and the specific detection of some subtypes (for example, H5 and H7). qRT-PCR assays are suitable for the screening of large numbers of samples, are very rapid, specific and much less expensive than conventional RT-PCR assays. A high degree of standardisation can be achieved between laboratories if standard protocols are followed. In particular, the selection of primers with appropriate specifications is most important. However, as influenza viruses are prone to genetic variation, there is also a need to constantly review the specifications for PCR primers and probes. The WHO and OIE Avian Influenza Reference laboratories undertake this role. For example, an assay that is optimised for the detection of the H5 subtype in one geographical region may not give optimal sensitivity in another. The laboratory at AAHL, Geelong, will advise of primer specifications that are most suited to the detection of viruses in the Australasian region.

When undertaking testing for the detection of Type A influenza viruses, samples should first be screened in a proven pan-reactive Type A assay (usually based on detection of sequences in the matrix (M) gene). Samples that react in the group assay should subsequently be tested in assays using primers specific for the H5 and H7 genes. If small numbers of samples are being tested, each of the assays can be run concurrently.

NOTE: the following assays have been included as they are in widespread use in veterinary laboratories in Australia and New Zealand but have yet to receive SCAHLS endorsement

1.5.1.1 Type A Influenza Virus qRT-PCR (Matrix) assay

This assay is designed to detect a segment of the matrix gene and consists of 2 separate stages – extraction of RNA from the test specimen and amplification and detection of viral RNA in the qRT-PCR. The details are as follows:

(a) RNA extraction

(i) Materials and equipment:

• RNA extraction kits * – suitable for extraction of viral RNA from viral transport medium, tissue homogenates and faecal samples;

- Various molecular biology grade chemicals such as 2-mercaptoethanol, 70% ethanol and isopropanol depending on the kit used;
- Microcentrifuge, adjustable speed up to 13,000 rpm;
- Orbital Mixer;
- 10, 200 and 1000 µL adjustable pipettors;
- Sterile, RNase free, aerosol resistant pipettor tips;
- Disposable gloves

***** Most laboratories now use commercial kits for the purification of viral RNA from a range of different sample types. These are typically based on mini column or magnetic bead technology. While there are differences in the yield and purity of the viral RNA extracted by some kits, individual laboratories should evaluate the kits available to determine the reagents that provide the best results in the specific environment. Once the viral RNA has been extracted and purified, it is ready for amplification in the qRT-PCR or can be stored at -20°C until tested.

(b) Quantitative ('Real-time') reverse transcription-PCR (qRT-PCR)

While there are some components of this assay that may be customised in an individual laboratory (see ** below), there are several components that must not be altered if optimal assays performance and inter-laboratory agreement is to be achieved. The various components are as follows:

(i) Materials and equipment.

- One-step qRT-PCR kit** including:
  - reverse transcriptase and DNA polymerase;
  - master mix
- Nuclease-free water
- Viral RNA (extracted from specimens or controls)
- Specific forward and reverse primers (18 µM) (see specifications in (ii) below)
- Specific Taqman® probes (5 µM) (see specifications in (ii) below)
- 2, 10, 200 and 1000 µL adjustable pipettors
- Sterile 0.5 mL microcentrifuge tubes
- Sterile, RNase-free aerosol resistant pipettor tips
- Powder-free disposable gloves
- Orbital Mixer
- Real-time PCR thermocycler
- 96-well optical reaction plate or PCR microtubes (depending on thermocycler)
- Adhesive covers for 96-well optical plates
- Benchtop centrifuge with 96-well plate adaptors (or microfuge if tubes are used)

****** Several commercial one-step qRT-PCR kits are available. However, some have superior performance that increases the sensitivity of the assay. These differences are believed to be mostly due to enhanced efficiency of the reverse transcriptase and to a lesser extent the DNA polymerase. Specific reagents should be evaluated in an individual laboratory by testing in parallel with reagents used in the original published method[52] to confirm suitability for routine use.

(ii) Primer and probe sequences.

Type A Matrix gene:
This is a generic assay to detect Influenza virus A viruses from different species, including avian, equine and/or other species, as appropriate.

Forward primer: IVA D161M Sequence: 5'-AGATGAGYCTTCTAACCGAGGTCG-3'
Reverse primer: IVA D162M Sequence: 5'-TGCAAANACATCYTCAAGTCTCTG-3'
TaqMan probe: IVA Ma Sequence: 5'FAM-TCAGGCCCCCTCAAAGCCGA-TAMRA3'

Alternate Type A Matrix gene:
This assay detects different region of influenza A virus matrix protein gene. The assay is used as a back-up for general Type A Matrix gene assay above.

Forward primer: IVA D233f Sequence: 5'- AAGACCAATCCTGTCACCTCTGA -3'
Reverse primer: IVA D227r Sequence: 5'-AAGCGTCTACGCTGCAGTCC -3'
TaqMan probe: IVA D231 Sequence:
5'FAM-TTTGTATTCACGCTCACCGTGCCCA-TAMRA3'

The cut-off values for the Type A Matrix gene TaqMan® assay are as follows:

Ct <38 = positive;
Ct 38 to 40 inconclusive, needs further investigation;
Ct >40 = negative

(iii) Internal control
The use of an internal control is recommended. A number of different options may be used. These provide some indication that RNA has been successfully extracted from the specimen and also provide an indication that the assay is not significantly affected by inhibitors in the test specimen. Before routine use, the chosen internal control system should be evaluated to show that it does not significantly affect the performance of the assay.

(iv) Method

- Prepare the primer/probe mix by mixing together equal volumes of primers D161M and D162M and probe Ma (FAM). This may be prepared in advance of running the assay. Dispense and store at -20°C.
- Determine the number of samples that will be tested, including positive and negative controls. Make provision for several 'no template control' reactions in addition to the test samples.
- Calculate the total volume of reagents required.
- Prepare a master mix for the TaqMan® assay, based on volumes required for the total number of reactions. Mix well. The volumes of the individual components for a single reaction are as follows:
    - Nuclease-free water 2.75 µL
    - 2x qPCR master mix 12.5 µL
    - 40x RT and Polymerase mix 0.625 µL
    - Type A (Matrix) primer probe mix 3.75 µL
    - Internal control primer probe mix 0.375 µL

Total volume 20 µL

- Dispense 20 µL of master mix into the appropriate wells (or tubes) in a 96-well optical reaction plate.
- Carefully dispense 5 µL viral RNA into each test well. Do not add any RNA to 'no template control wells'.
- Seal the plate with an optical adhesive cover.
- Centrifuge the plate (or tubes) briefly in a bench centrifuge with 96-well plate adaptors, to ensure that the complete reaction mix is at the bottom of the well or tube.
- Place the plate (or tubes) in the real-time PCR thermocycler and run with the following parameters:
    - 30 min at 48°C (reverse transcription);
    - 10 min at 95°C (hot-start Taq polymerase activation);
    - 45 cycles of 15 sec at 95°C;
    - 1 min at 60°C (target amplification and acquisition of fluorescence data).

Note: The times and temperatures for the reverse transcription and polymerase activation may need to be varied with different master mixes

- Analyse the results using software supplied with the thermocycler.

Note: Standard curves will need to be determined for different instruments and platforms to establish the linear range for assay sensitivity and limits of detection. As a guide, with a threshold set at 0.05, the 'cut-off' values for the Type A (matrix gene-specific) TaqMan® assay are

o Ct <37 = positive;
o Ct 37 to 40 inconclusive, needs further investigation;
o Ct >40 = negative.

1.5.1.2 Sub-type Specific Influenza Virus qRT-PCR assays

The assays for the respective subtypes are run under the same conditions as the Type A matrix assay by substituting the primers and probes as follows:

(a) Subtype H5 Multiplex assay
The multiplex TaqMan assay consists of two sets of primers and probes targeting 2 different regions (C-terminus and N-terminus) of HA gene.

A. C-terminus
Forward primer: IVA D148H5 Sequence:
5'-AAACAGAGAGGAAATAAGTGGAGTAAAATT-3'
Reverse primer: IVA D149H5 Sequence: 5'-AAAGATAGACCAGCTACCATGATTGC-3'
TaqMan probe*: IVA H5a Sequence:
5'FAM-TCAACAGTGGCGAGTTCCCTAGCA-TAMRA3'

B. N-terminus
Forward primer: IVA D204f Sequence: 5'-ATGGCTCCTCGGRAACCC-3'
Reverse primer: IVA D205r Sequence: 5'-TTYTCCACTATGTAAGACCATTCCG-3'
TaqMan probe*: IVA D215prb Sequence: 5'FAM-ATGTGTGACGAATTCMT-MGBNFQ3'

C. H5 HA Cleavage region back-up assay for H5 Multiplex assay
This assay is used as a back-up assay for the above two assays.

Forward primer: IVA D207f Sequence: 5'-GGGGAATGCCCCAARTATGT-3'
Reverse primer: IVA D213r Sequence: 5'-gTCAATRATYGAGTTGACCTTATTGGT-3'
TaqMan probe: IVA D218prb Sequence: 5'FAM-CATTCCCTGCCATCC-NFQMGB

The cut-off limits for the H5 Multiplex TaqMan® assay are as follows:

Ct <38 = positive;
Ct 38-40 = inconclusive, needs further investigation;
Ct >40 = negative

(b) Subtype H7

Forward primer: IVA D168H7 Sequences: 5'-GGATGGGAAGGTYTGGTTGA-3'
Reverse primer: IVA D169H7 Sequence: 5'-CCTCTCCTTGTGMATTTTGATG-3'
TaqMan probe: IVA H7-AUS Sequence: 5'FAM-TGAAACCATACCACCCA-MGBNFQ3'
The limits of detection for the H7-specific TaqMan® assay are as follows:

Ct <37 = positive;
Ct 37 to 40 inconclusive, needs further investigation;
Ct >40 = negative

More information on primers and probes and assay performance can be obtained by contacting the Australian Animal Health Laboratory (see Part 3.5).

1.6 Rapid detection of Type A Influenza viral antigens by commercial kits

A range of rapid diagnostic tests that are designed to detect influenza viral antigens has been developed and are now available from commercial sources. These 'point of care' (POC) or 'pen-side' tests do not have the sensitivity of virus isolation or PCR but are usually able to detect viral antigens in birds that are clinically affected with AI. There are a range of different test formats, usually in a 'dip-stick' or a lateral flow immuno-chromatographic strip style, some of which are Type A reactive and others that are subtype (usually H5) specific. They should be regarded as 'flock' tests and while a negative result does not exclude AI, a positive result can be very suggestive. However, these kits cannot be used alone to provide confirmation or exclusion of influenza. When these tests are used, especially in a field situation, the usual range of samples should be promptly submitted to a diagnostic laboratory for further investigation, irrespective of the initial results. Further details are provided in Part 3 – "Reagents and test kits".

2. Serology

Serological tests can be used to detect antibodies in infected animals or to identify virus isolates. Serology is of little use in the diagnosis of acute HPAI in individual affected birds but should still be undertaken on surviving birds as HPAI viruses often enter domestic poultry populations as LPAI and become virulent with subsequent passage. Further, if early cases have not been detected, when there is slow spread of virus (for example, among caged layers) it is possible that there may be some birds that have been infected but recovered. Serology may also be necessary to demonstrate freedom of infection following an outbreak. General surveillance is best undertaken using a generic Type A influenza test such as the AGID or ELISA. The ELISA is more efficient and appropriate for large scale testing. Once the virus causing an outbreak has

been identified, the subtype-specific HI test can be employed to confirm that birds that are seropositive in a Type A group test have been exposed to a virus of the same subtype.

Commercial ELISA kits that detect antibodies against the nucleoprotein are available but it is important to ensure that they are 'fit for purpose' for the intended type of testing. At this time, none has been validated for use in Australia. The neuraminidase-inhibition test has been used to identify the AI neuraminidase (N) type of isolates and to characterise the antibody in infected birds. The procedure requires specialised expertise and reagents and is usually done in a Reference Laboratory.

2.1 Agar Gel Immunodiffusion

Chickens and turkeys can be tested for antibodies against influenza viruses or a presumptive influenza virus can be identified as a Type A isolate in agar gel immunodiffusion (AGID) tests by demonstrating the presence of the nucleocapsid or matrix antigens, both of which have epitopes that are common to all influenza A viruses. The AGID has been of little use in testing sera from wild birds as they generally do not produce detectable levels of precipitating antibody, however many of the Asian H5N1 viruses do generate antibodies in wild birds that are detectable in the AGID. Minor variations in the procedure are practised in different laboratories but 8% NaCl is necessary for the precipitation of avian antibodies. To maximise the sensitivity of the test, the antigen and antiserum must be titrated to ensure that they are used at the highest possible working dilution, while maintaining good balance of the reference precipitin line midway between the wells.

2.1.1. Preparation of Medium

(a) Add 1 g of Agarose to 100 mL of phosphate buffered saline containing 8% NaCl. The recipe for 'high salt' buffer solution as follows:

NaCL	80.0 g
KCl	0.2 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.2 g
Sodium azide, NaN_3	1.0 g

Add 1 L of type 1 (distilled) water and adjust pH to 7.2.

(b) Heat in a microwave oven on the highest power available for 10 min.

(c) Cool to 56°C in a water bath and pour 15 mL into 9 cm diameter plastic petri dishes and allow to set.

2.1.2. Antigen and Reference Antiserum

These reagents are available from the reference laboratory (see Part 3, "Reagents and Test Kits").

2.1.3. Agar Gel Immunodiffusion Test

(a) Cut wells in the agar in the petri dishes. Patterns of seven wells, one central and six peripheral, in a hexagonal pattern, are cut exactly perpendicular to the agar surface. The

agar plugs should be removed carefully, so that well walls and the agar surface are undamaged and not touched. The wells should be 5–6 mm in diameter and 2 mm apart.

(b) The peripheral well of each pattern, that is, the well closest to the perimeter of the dish, is identified as well No. 1 and wells 2–6 counted clockwise from it. Six patterns should fit around the perimeter of a dish. The space left in the centre, which would accommodate a seventh pattern, must not be used.

(c) Using 50 μL of reagent per well, load reference antigen in the central well and reference serum in wells 1, 3 and 5. Specimens to be tested for antibody or antigen should be loaded into wells 2, 4 and 6. This arrangement is important as it prevents reactivity between different patterns. A known positive and negative serum should be included as controls in each test. Any unused wells on the plate should be filled with buffer solution.

2.1.4. Reading and interpretation of results

(a) Read the test after holding the plates at room temperature or 37°C in a humidified atmosphere for 24 h. Negative or suspicious reactions should be re-examined at 48 h.

(b) Examine the petri dishes in subdued light with the dish illuminated from beneath by a narrow beam of light (for example, an egg-candling light) at an angle of about 45°.

(c) Antibody activity in the test sample turns the reference lines within the peripheral hexagon and antigen causes reference lines to turn outward between peripheral wells. Quantitation is done by comparison of the test with the reference lines.

(d) The following scale is used for reporting results (Table 1.) Pictures of the precipitation lines in an AGID are provided in Della-Porta et al (1983)[55].

Table 1. Scale for reporting the results of the agar gel immunodiffusion test

Antibody	*Antigen*	*Comments*
Neg	Neg	No reaction detected – the reference lines do not bend and extend into the centre of the test well.
>3	XS	Denotes sample stronger reactivity than reference. Care should be taken with sera of very high antibody titre that inhibit precipitation on adjacent reference lines. They should be retested at dilutions.
3	+++	Denotes sample reactivity equivalent to reference
2	++	Denotes sample weaker in reactivity than reference but producing a continuous line across the face of the test well
1	+	A distinct turn on the end of the reference line
Non-specific		Lines of precipitation which either cross or fail to establish a line of identity with the reference line

2.2 Blocking ELISA

The specificity of a blocking ELISA (bELISA) is determined by the reactivity of the monoclonal antibody (mAb) that is the key component of the assay. There are now both Type A group

reactive assays that will detect antibodies to any Type A influenza virus and some assays that are subtype (for example, H5) specific. The method included below is for a Type A group reactive assay and is likely to detect antibodies in any avian species. While the test has been fully validated for use in chickens and turkeys, there is uncertainty of the threshold values for positive results in wild bird species. Nevertheless, it is believed that negative results in any bird species will indicate a lack of exposure to Type A influenza viruses. The bELISA has advantages of both very high sensitivity and specificity and is the test of choice for large scale testing. There are also several commercially available ELISAs that are believed to be specific for the detection of antibodies to H5 viruses but none has been evaluated or approved for use in Australia.

The assay that is described below uses a mAb that reacts with an epitope on the nucleoprotein that is conserved in all type A influenza viruses. All reagents and the full protocol are available from the Australian Animal Health Laboratory.

2.2.1 Reagents

Care must be taken to ensure that all reagents are stored according to the manufacturer's instructions unless specified otherwise in this document.

(a) Antigen

The assay employs 96-well flat-bottomed plates or strips that are coated with recombinant nucleoprotein antigen expressed in *E coli*. ELISA plates may be coated with antigen at 37°C on the day of the test or on the day before the test and left at room temperature or 4°C overnight.

(b) Control sera

These consist of a high positive control, a low positive control and a negative control serum. All 3 control sera are used at a dilution of 1/10. These should each be stored at or below –20°C as 'undiluted' serum in small volumes (appropriate to the usual number of plates being used) so they are thawed and used once only. The dilutions and expected results for the positive controls will be supplied by AAHL with each batch of reagents.

***(c) Monoclonal Antibody* (mAb)**

The monoclonal antibody used is an anti-Influenza A nucleoprotein mAb supplied by AAHL.

(d) Conjugate

The conjugate is commercially available horseradish peroxidase-conjugated goat anti-mouse IgG (H&L). This conjugate is initially titrated to determine the optimal dilution by standard calibration procedures using known batches of antigen, antisera and conjugate.

2.2.2 Solutions

(a) 0.05M Carbonate Buffer (pH 9.6)

This is the buffer used to dilute antigen to coat ELISA plates. The high pH improves the binding of protein to the well surface.

Na_2CO_3 1.59 g
$NaHCO_3$ 2.93 g
Make up to 1 litre with distilled water and adjust pH to 9.6

(b) Phosphate Buffered Saline, pH 7.2 (PBS)

NaC1 8.0 g

KC1 0.2 g
Na_2HPO_4 1.15 g
KH_2PO_4 0.2 g
Make up to 1 litre with distilled water and adjust pH to 7.3.

(c) Washing solution – PBST
To one litre of PBS add 0.5 mL of Tween 20. (0.05% Tween 20)
Washing solution should not be stored longer than 1 week.

(d) Sample Diluent – PBST-SM
1.0 g skim milk powder
100 mL PBST (washing solution)
Do not store this solution for longer than 2 days.

(e) Substrate Solution
The substrate is prepared from the TMB stock, citrate-acetate buffer and H_2O_2 as follows:

i) TMB Stock Solution.
Dissolve 0.101 g of TMB (Sigma T-2885) in 10 mL of DMSO (Sigma D-5879) to make a 42 mM solution. Store in 1 mL volumes at 4°C. Note: TMB is very sensitive to light.

ii) Substrate Buffer (0.1M Citrate - acetate buffer pH 6.0)
1 M Sodium Acetate 100 mL
1 M Citric Acid 1.5 mL
Adjust pH to 6.0 with 1 M Citric Acid

iii) Activated Substrate Solution
Add 2 mL of substrate buffer concentrate to 18 mL of distilled H_20. Volumes can be adjusted according to the number of plates tested. To 20 mL of the substrate buffer add 0.2 mL of TMB substrate, 2.5 µL of 30% H_20_2 and mix well. It is important to use a new pipette trough for mixing and dispensing the activated substrate mixture.

(f) 1M Sulphuric Acid (stopping solution)
Add 27.8 mL of concentrated H_2SO_4 to 472.2 mL of distilled water.
NOTE: ALWAYS ADD THE ACID TO THE WATER

2.2.3 Materials and Equipment

(a) Disposables
- 96-well flat-bottomed high binding ELISA plates or strips – (Nunc Maxisorb, Cat 655061).
- 96-well microtitre plates – low- or non-binding to dilute samples.
- tips for micropipettors

(b) General equipment and glassware
- Single channel micropipettors:
  5-40 µL
  40-200 µL
  200 µL – 1 mL
- Multichannel micropipettors (8 and 12 channel) 5-50 µL and 50-200 µL
- Multistepper pipettes (50/100/150/200 µL)

- Sterile troughs for reagents
- Automatic pipettor for serological pipettes
- Sterile glass or plastic pipettes (5 and 10 mL)
- Sterile vessels for diluents and solutions
- Container(s) for waste disposal (especially pipette tips)

(c) Major Equipment
- Automatic 96-well plate washer
- ELISA reader
- Plate shaker
- 37°C incubator or warm room
- Computer

2.2.4 Test Procedure

ELISAs are biological tests that must be carried out under defined conditions for optimal performance. It is essential to follow the instructions for handling and use of reagents as described in this document. Apart from reagents, the most important factor to influence the performance of the test is temperature. Particular temperatures are specified at various stages and these must be achieved throughout the time interval stipulated. As these assays and various steps are conducted over relatively short time periods, it is essential to ensure that the reagents and materials are adjusted to the relevant temperature prior to use in the test.

(i) Dilute the antigen to the appropriate dilution in 0.05M carbonate buffer (pH 9.6). The dilution of antigen to be used is determined by prior titration, to give an optical density of 0.8 - 1.5 with both blank and normal chicken serum, in the presence of optimal dilutions of mAb and conjugate.

(ii) Coat ELISA microplates with antigen by adding 50 µL per well. Tap or gently shake plates to ensure even dispersal of the antigen over the surface.

(iii) Incubate the plates at 37°C for 60 min or hold plates overnight (at either room temperature, approximately 20 - 25°C, or at 4°C in a humidified chamber).

(iv) Wash plates 3 - 5 times with PBST and tap dry. This should be done immediately before the addition of the test samples and control sera.

(v) Dilute test sera and control sera 1/10 in sample diluent (PBST-SM). Add 50 µL of each test and control serum to the antigen coated plates in duplicate. Include at least two 'blank' wells, with sample diluent only, on each plate.

(vi) Ensure plates have a tight fitting lid. Incubate at 37°C for 30 min on a plate shaker at approximately 500 rpm (for example, Flow, speed 5-6).

(vii) Dilute the mAb to the appropriate dilution in sample diluent. Without washing the plates add 50 µL of diluted mAb to each well.

(viii) Place tight fitting lids on plates and incubate at 37°C for 30 min on a plate shaker at approximately 500 rpm (for example, speed 5-6 on a Flow shaker).

(ix) Wash plates 3 - 5 times with PBST.

(x) Dilute conjugate (horseradish peroxidase conjugated goat anti-mouse IgG (H+L)) in sample diluent (PBST-SM) and add 50 µL to each well.

(xi) Place tight fitting lids on plates and incubate at 37°C for 30 min on a plate shaker at approximately 500 rpm (for example, speed 5-6 on a Flow shaker).

(xii) Wash plates 3 - 5 times with PBST.

(xiii) Develop plate by adding 50 µL of freshly prepared, warmed, activated substrate solution (H_2O_2/TMB) to each well.

(xiv) Incubate at room temperature for approximately 10 min, protected from light. In some circumstances (for example, cold conditions) more consistent results may be obtained by incubating at 37°C.

(xv) Stop the enzyme reaction by adding 50 µL of 1M sulphuric acid to each well. Shake the plate to mix well.

(xvi) Read the optical density (OD) of each well at 450 nm on the plate reader.

(xvii) The final result, or percentage inhibition (PI), is calculated as the difference in reactivity of the test sample compared to the negative control. If it is necessary to calculate the results manually, the formula used is:

$$\text{Percentage inhibition} = 100 - \left[\frac{\text{Mean OD Sample}}{\text{Mean OD Mab control}} \times 100 \right] \%$$

2.2.5 Interpretation of results

Test 'cut-off' criteria have been established for chicken and turkey sera as follows:

Percentage inhibition < 40%	Negative
Percentage inhibition between 40% and 60%	Inconclusive
Percentage inhibition > 60%	Positive

A positive result in this test indicates that the bird has been infected at some time with a group A Influenza virus. A negative result indicates that it is unlikely that the bird has ever been infected by a group A Influenza virus. An inconclusive result indicates that retesting or re-sampling is required. The bELISA has been extensively validated on chickens and turkeys, but there is some doubt about the cut-off threshold for ducks and wild birds. However, a negative test result usually indicates that a bird has not been infected with a Type A influenza virus. Samples positive or doubtful in the bELISA are usually tested by the HI test to determine the H subtype of the virus involved. As the HI test is essentially 'H' type-specific, it is essential to test samples separately in HI tests with antigen from the relevant subtypes, especially H5 and H7.

2.3 Haemagglutination Inhibition Test

The HI test is specific to each of the 16 individual H subtypes of Type A influenza viruses. This specificity is determined by the haemagglutinin antigens on the viral surface. Therefore, when the test is conducted, to achieve the appropriate specificity, it is necessary to use different antigens (derived from the homologous viruses) for each of the 16 H subtypes and, in some instances, viruses within a haemagglutinin subtype.

The method described below is a HI test carried out in U-bottom microtitre plates, with 25 µL of serum and antigen, 4 haemagglutinating units (HAU) of antigen and 50 µL of a 0.5% suspension of chicken red blood cells (CRBC). Variations to this method are commonly used, such as V-bottom microtitre plates, 8 HAU of antigen, 25 µL of a 1.0% suspension of CRBC and different temperatures for incubations. However, such variations can lead to differences in interpreting results and laboratories should standardise test protocols to minimise variations between tests.

2.3.1 Reagents

Care must be taken to ensure that all reagents are stored according to the manufacturer's instructions unless specified otherwise in this document.

(a) Antigen

The assay employs infectious allantoic fluid as the antigen. Influenza virus is grown in the allantoic sac of 9-11 day-old embryonated chicken eggs, the allantoic fluid harvested and stored at -80°C in appropriate volumes. The exact conditions for growth will differ for different isolates. Antigens can be inactivated by chemical or physical means for safety reasons.

(b) Control sera

These consist of a high positive control, a low positive control and a negative control serum. All 3 control sera are titrated so that the titre of each can be recorded. These should each be stored at or below –20°C as 'undiluted' serum in small volumes (appropriate to the usual number of plates being used) so they are thawed and used once only. The dilutions and expected results for the positive controls will be supplied by AAHL with each batch of reagents.

2.3.2 Solutions

(a) Phosphate Buffered Saline, pH 7.2 (PBS)

Refer to Section 2.2.2b (above)

(b) **Receptor Destroying Enzyme** (RDE).

Used for treatment of problem serum samples. Supplied by Welcome Reagents Ltd.

2.3.3 Materials and Equipment

(a) Disposables

- 96-well V- or U-bottom polystyrene plates
- tips for micropipettors

(b) General equipment and glassware

- Single channel micropipettors:
  - 5-40 µL
  - 40-200 µL

200 µL – 1 mL
- Multichannel micropipettors (8 and 12 channel) 5-50 µL and 50-200 µL
- Multistepper pipette (50/100/150/200 µL)
- Sterile troughs for reagents
- Automatic pipettor for serological pipettes
- Sterile glass or plastic pipettes (5 and 10 mL)
- Sterile vessels for diluents and solutions
- Container(s) for waste disposal (especially pipette tips)

(c) Major Equipment
- 37°C incubator or warm room

2.3.4 Preparation of the chicken red blood cell suspension

Blood for the preparation of a chicken red blood cell (CRBC) suspension should come from SPF chickens, but if this is not possible, use blood from known antibody-free birds. Red blood cells from species other than chickens are sometimes used but CRBCs are the cell of choice. Ideally blood from several birds should be pooled. Blood in Alsever's solution may be stored at 4°C for several weeks and as washed cells for up to one week. Do not use cells that are obviously haemolysed. Cells should be prepared as follows:

(a) Collect whole blood in a syringe containing Alsever's solution (0.5–1.0 mL in a 10 mL syringe). Quickly transfer the blood to a bottle containing more Alsever's solution so that the final ratio is one part of blood to 5 parts Alsever's solution and mix well.

(b) Centrifuge the cells at 1500 g for 5 min and decant the supernatant, removing the white blood cell layer on top of the red blood cells.

(c) Gently resuspend the cells in cool phosphate buffered saline (PBS) pH 7.3.

(d) Repeat the washing steps a minimum of three times.

(e) Resuspend the cells in a small volume of PBS and determine the packed cell volume using a microhaematocrit or spectrophotometer.

(f) Prepare the cells as a 10% (v/v) suspension and store at 4°C. Dilute this stock for use in the HA and HI tests.

Note: The determination of the cell concentration is critical to the accuracy of test results. Variations in the cell concentration will affect the amount of virus used in the test.

2.3.5 Haemagglutinin Titration

This assay can be used to determine the titre of antigen in a virus stock prior to use in a HI test or can be used to screen specimens (for example, allantoic fluids) to detect the presence of haemagglutinating antigen. The titre of haemagglutinating (HA) antigen in a virus preparation is determined and standardised before each HI test as described below. Titrations of antigen should be done in duplicate as a minimum, however more replicates may be used.

(a) Place 25 µL of phosphate buffered saline (PBS) into each well of a 96-well U-bottom microtitre plate.

(b) Place 25 µL of the antigen (or suspect allantoic fluid for antigen detection) to be tested in the first well of a row. For titration of a known antigen, to obtain optimal accuracy, the antigen may be placed in the first well of several successive rows at a range of close starting dilutions, for example, 1:2, 1:3 & 1:5.

(c) Make serial 2-fold dilution of each row from the first well to well 11 (a 1:2048 dilution), mix thoroughly in each well and discard 25 µL from well 11. Column 12 will act as the cell control

(d) Add a further 25 µL of PBS to each well.

(e) Add 50 µL of freshly prepared 0.5% CRBC suspension to each well.

(f) Mix by lightly tapping the plate or placing on a microplate shaker and mix gently for 15-30 seconds.

(g) Cover and place the plate at room temperature (approximately 20-25°C) for at least 40 minutes or at 4°C for 45-60 minutes. The RBC control well should show a tight, small button after that time.

(h) The plates are read for HA activity. The haemagglutination in U-well plates can be read using a plate reading mirror while V-well plates are usually tilted at an angle of about 45 deg for about 1 min. In wells in which agglutination occurs, there will be no evidence of streaming of the RBCs across the wall of the well. The endpoint is the highest dilution at which complete haemagglutination occurs (that is, NO running of RBCs in V-well plates). Partial agglutination is scored as negative. There should be no agglutination in either the cell control (PBS only) or negative serum rows. The titre of the virus is the reciprocal of the highest dilution that gives complete agglutination (or, in V-well plates, the last well in which the cells do not run). If the highest dilution that gives complete agglutination is 1:256 the titre of the antigen is 256 haemagglutinating units (HAU)/25 µL.

(i) To find the dilution factor required for the test antigen divide the established HA titre of the antigen by the designated number of HAU required for the test (for example, four for avian influenza). For example, if the HA titre of the antigen is 256 and four HAU are required for the test, then 256/4 = 64, which is the dilution required to provide four HAU of challenge antigen per well.

2.3.6 The Haemagglutination Inhibition Assay

2.3.6.1 *Treatment of sera to remove non-specific agglutinins*

Serum from chickens may be tested for HI antibodies without treatment, but sera from species other than chickens may contain factors that agglutinate CRBC. These factors should be removed by adsorption of the serum with CRBC prior to testing. Add 5 µL of washed, packed CRBC to 25 µL of each serum, mix gently and leave at 4°C for at least 30 min. Add 75 µL of PBS to each serum to give a 1:4 dilution and pellet the CRBC by centrifugation at 1500 *g* for 5

min. Decant the adsorbed sera and test starting as a 1:4 dilution. In large tests, or tests in which results are critical, the back titration of the working dilution of antigen may be done before the addition of the antigen to the test sera.

2.3.6.2 *HI Test method*

(a) Place 25 µL of PBS in each well of a U-bottom microtitre plate.

(b) Place 25 µL of each test serum in the first well of separate rows, and 25 µL of the same serum in the last well (serum control) of the same row.

(c) Place 25 µL of known positive serum in the first well of a row (positive control).

(d) Place 25 µL of known negative serum in the first well of a row (negative control).

(e) Place 50 µL of diluted antigen in the first well of a row (back titration of antigen). Remove PBS from this well before adding antigen.

(f) Serially dilute all rows from the first to the second last well of each row and discard 25 µL from the wells in the second last column.

(g) Add 25 µL of antigen diluted to contain 4 HA units to each well except for those containing the serum controls and re-titrated antigen. Add 25 µL PBS to these wells.

(h) Tap plates gently or place on a microplate shaker and mix gently for 30 sec.

(i) Hold plates at room temperature for 30 min.

(j) Add 50 µL of a 0.5% suspension of CRBCs to each well and tap gently or place on a microplate shaker and mix gently for 30 sec.

(k) Cover and place the plate at room temperature (approximately 20-25◦C) for at least 30 min or at 4◦C for 45 min. The RBC in the wells containing the serum control (no virus) should show a tight, small button at that time.

(l) The plates are read for HA. The haemagglutination in U-well plates can be read using a plate reading mirror while V-well plates are usually tilted at an angle of about 45 deg for about 1 min. In wells in which there is inhibition of agglutination, there will be evidence of streaming of the RBCs across the wall of the well. The endpoint or titre of the serum is the highest dilution at which there is complete inhibition of haemagglutination. There should be haemagglutination of cells in the serum control wells.

(m) The controls are read as follows:
   (i) *Positive*: complete inhibition of buttoning and running of red cells to a known titre
   (ii) *Negative*: complete agglutination (no running or buttoning)
   (iii) Back *titrated antigen*: agglutination (no running) in the first three wells for AI, (that is, titre of 4 HAU) with RBC buttons and running of cells in all other wells.

   N.B. If these controls differ by more than one, 2-fold dilution from the expected value the test should be repeated, unless the result is unambiguous.

2.3.6.3 *Interpretation of results*

A serum is regarded as being positive for antibody if there is complete inhibition at a serum dilution of 1:16 (a titre of 2^4 or 16) or more against 4 HAU of antigen. If laboratories use 8 HAU in the HI test a serum is regarded as positive if it shows complete inhibition at a serum dilution of 1:8 (a titre of 2^3 or 8) or more.

2.3.6.4. *Non-specific reactions*

These are occasionally observed, usually at a titre of less than 1:64. In such cases the reacting serum samples may be treated overnight with Receptor Destroying Enzyme (RDE). This treatment involves diluting the serum samples 1:4 with RDE (Welcome Reagents Ltd) and incubating at 37°C overnight. The mixture is then inactivated by heating at 56°C for 30 min. This treatment usually completely abolishes most non-specific HI titre. If titres still persist in a few sera after this treatment a further 20 serum samples from the flock concerned should be tested. Samples should be tested concurrently in the cELISA, where positive results would be expected if the reactions are specific to an AI virus. All the available evidence from the flock, the clinical signs, gross pathology and virological findings must be taken into account before a diagnosis is made.

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Part 3: Reagents and Test Kits for Avian Influenza Diagnosis in Australia and New Zealand

The list of suppliers provided below may not be exhaustive but includes all materials (complete kits and other reagents) that have been evaluated and found to be suitable for use under Australian or New Zealand conditions. These reagents and kits have been approved only for use in Australia or New Zealand for the purposes and within the limits described below. Other suppliers who have materials that may be used for the purposes described below are welcome to submit reagents and kits evaluation by contacting the Executive Officer, SCAHLS <www.scahls.org.au>.

1 Rapid Antigen Detection Kits

Many tests for the rapid detection of influenza viruses in clinical specimens have been recently developed and marketed. These were initially developed for use on humans and are based on ELISA or immunochromatographic detection systems, but have now been adapted, or especially developed, for use in animals. Some of the kits available are Directigen (Becton Dickinson), Flu OIA (Biota), Anigen (Life Bioscience), Flu Detect (Synbiotics) and Binax (Inverness Medical). The primary advantage of these tests is that antigen can be detected in swabs in as little as 15-30 min. The main disadvantages are their lack of analytical sensitivity, as they require large amounts of virus to give a positive result, and the cost of the kits. Also, most of the kits will detect only influenza A viruses and provide no subtype identification. Several kits have been evaluated for use in birds and have shown good sensitivity and specificity on specimens from chickens experimentally infected with H5N1 HPAI virus.

Despite promising results on specimens from experimentally infected chickens care should be exercised in the use of these kits. False positive reactions are common with cloacal swabs with a high faecal load and tracheal swabs containing mucus. The lack of sensitivity means that the kits will not consistently detect LPAI viruses in healthy birds and should be used only on specimens collected from birds showing clinical disease. Regardless of the result of a rapid test, all suspect cases of HPAI should be subject to a laboratory investigation. Although a comparative evaluation of many of these kits has been published[53], as yet none of these kits has been endorsed by SCAHLS for routine use in Australia.

2. Blocking ELISA Reagents

All key reagents (Antigen, monoclonal antibody, control sera and conjugate) for the Type A influenza bELISA are available from CSIRO, AAHL, Geelong Victoria. Peroxidase conjugated antimouse antibody from commercial sources may also be used. All reagents for the bELISA are available either in complete ‘kit’ form or individually. Supply of reagents in kit form is recommended as all reagents have been calibrated for optimal reactivity. No other antibody kits have been evaluated for use in Australia.

3. HI reagents

Inactivated antigens, and positive and negative control sera for each H type are available from CSIRO AAHL. Monospecific antisera for virus identification and inactivated antigens for use as PCR positive controls are also available.

4. AGID Reagents

AGID antigen and antiserum are available from CSIRO, AAHL.

5. National Reference Laboratories

5.1 Australia:

CSIRO Australian Animal Health Laboratory
5 Portarlington Rd
East Geelong, Vic 3219

Postal address: Private Bag 24
Geelong 3220

Tel 61 3 5227 5000
Fax 61 3 5227 5555

5.2 New Zealand:

MAF Biosecurity New Zealand
Investigation and Diagnostic Centre
66 Ward Street
Upper Hutt 5018

Postal address: PO Box 40742
Upper Hutt 5140

Tel 64 4 894 5600
Fax 64 4 894 4973