Published in final edited form as:

Nat Protoc. 2014 November ; 9(11): 2663–2681. doi:10.1038/nprot.2014.180.

Influenza A Virus Isolation, Culture and Identification

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SUMMARY

Influenza A viruses (IAV) cause epidemics and pandemics that result in considerable financial burden and loss of human life. To manage annual IAV epidemics and prepare for future pandemics, improved understanding of how IAVs emerge, transmit, cause disease, and acquire pandemic potential is urgently needed. Fundamental techniques essential for procuring such knowledge are IAV isolation and culture from experimental and surveillance samples. Here, we present a detailed protocol for IAV sample collection and processing, amplification in chicken eggs and mammalian cells, and identification from samples containing unknown pathogens. This protocol is robust, and allows for generation of virus cultures that can be used for downstream analyses. Once experimental or surveillance samples are obtained, virus cultures can be generated and the presence of IAV can be verified in 3–5 days. Increased time-frames may be required for less experienced laboratory personnel, or when large numbers of samples will be processed.

INTRODUCTION

Influenza A viruses (IAVs) cause seasonal epidemics that are responsible for 3–5 million severe human cases and 250,000 deaths worldwide annually¹, and sporadic pandemics that can induce an even higher disease burden and loss of human life. Seasonal epidemics are caused by antigenic drift, a process facilitated by a combination of immune pressure and an intrinsically high IAV mutation rate. Specifically, when the hemagglutinin (HA) protein of a circulating IAV acquires mutations that enable immune evasion, such a virus can spread efficiently in humans that lack preexisting antibodies against the antigenically drifted

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AUTHOR CONTRIBUTIONS STATEMENTS

AJE, GN, and YK wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

variant. For this reason, seasonal IAV vaccines must be reformulated every 1–3 years. Pandemic IAVs emerge when novel avian IAVs, or reassortant IAVs possessing genes derived from avian, human, and/or swine viruses – for which most humans lack immunity – are transmitted to humans from reservoir species (i.e., birds and swine). Five IAV pandemics occurred during the 20th and 21st centuries (i.e., in 1918, 1957, 1968, 1977, and 2009); the worst of these was the 1918 Spanish influenza, which was responsible for 50 million deaths².

IAVs are diverse. Virus subtypes are categorized based on the antigenic properties of the HA and neuraminidase (NA) virion surface proteins, and to date, 17 HA subtypes (e.g., H1, H2 etc.) and 10 NA subtypes (e.g., N1, N2 etc.) have been identified. All HA and NA subtypes are found in avian species (with the exception of H17 and N10, which have been detected only in bats³), whereas viruses of only a few subtypes are found in swine. Currently, viruses of the H1N1 and H3N2 subtypes circulate in humans; however, since 1997, highly pathogenic avian influenza (HPAI) viruses of the H5N1 subtype (H5N1-HPAI) have occasionally transmitted from birds to humans, and to date such zoonotic events have caused at least 667 clinically confirmed human cases and 393 deaths (58.9% mortality)⁴. The steep mortality rate associated with human H5N1-HPAI infections is alarming, but the lack of efficient human-to-human transmission has, so far, prevented these viruses from causing a pandemic. Nonetheless, recent reports indicate that only a few mutations may be needed for H5N1-HPAI viruses to acquire transmission capability in mammals, and some of these mutations are already present in human isolates^{5–7}. In the spring of 2013, viruses of the H7N9 subtype emerged in humans in China⁸, and the ongoing epidemic comprises >400 infections⁹. These H7N9 viruses already are capable of limited transmission in humans¹⁰ and in mammalian infection models^{11–14}, suggesting that they possess pandemic potential.

Surveillance to detect IAVs in human and animal populations is necessary to guard against seasonal epidemics, as well as novel and potentially devastating pandemic viruses. In particular, routine surveillance is conducted in humans year-round, and is critical for monitoring influenza activity, evaluating vaccine efficacy, and to assist in vaccine strain selection for upcoming IAV seasons. Surveillance is also performed in animal reservoirs to identify potential pandemic threats. Fundamentally, a successful surveillance program requires the ability to isolate, amplify, and identify IAV from various sources. In this protocol, we describe standard procedures for IAV surveillance sample collection, virus culture, and virus identification, which have been developed over decades of research. The protocol is presented in three parts (a flowchart of the protocol is depicted in Figure 1), focusing on sample collection and processing (**Part 1**); virus culture in either embryonated chicken eggs or mammalian cells (**Part 2**); and verification of the presence of IAV using hemagglutination (HA) assays and reverse transcriptase polymerase chain reaction (RT-PCR) analysis (**Part 3**).

Protocol Design

It should be noted that this protocol is primarily designed for use in research, and is not a clinical protocol. In addition, it is important to appreciate that the described techniques are applicable not only for surveillance samples, but also for experimental samples generated

during basic research. For additional information extending beyond the scope of the current protocol, readers are referred to the World Health Organization (WHO) Global Influenza Surveillance Network (GISN) “Manual for the Laboratory Diagnosis and Virological Surveillance of Influenza”¹⁵, the WHO “Manual on Animal Influenza Diagnosis and Surveillance”¹⁶, the WHO informational document describing the molecular diagnosis of influenza in humans¹⁷.

Sample collection—Most IAV surveillance samples are collected from humans exhibiting respiratory disease symptoms and are processed and analyzed in local, national, and global laboratories throughout the world (for examples, see references^{18–20}). However, human samples also may be collected and processed by research laboratories in the context of clinical trials, or for occupational medical surveillance programs. Animal surveillance samples are collected from swine, poultry, wild birds and other species to advance overall knowledge of IAV epizootiology, pinpoint the source(s) of emerging or pandemic viruses, and for other medical, veterinary, or experimental purposes. The collection and processing of the following types of samples are described in this protocol:

- **Swab samples** from humans (nasal, nasopharyngeal, or oropharyngeal); avian species (oropharyngeal, tracheal, cloacal, or fecal); and non-human, mammalian species (nasal, naso- or oropharyngeal, tracheal, rectal, or fecal)
- **Liquid samples** from environmental sources (e.g., water from lakes or troughs in live poultry markets); experimentally infected animals (e.g., nasal wash, bronchoalveolar lavage); and humans (nasopharyngeal aspirates, naso- or oropharyngeal gargles, bronchoalveolar lavage, sputum, or tracheal aspirates)
- **Tissue samples** from experimentally sacrificed animals, or from slaughtered or dead animals (tissues may include blood, respiratory organs [nasal turbinate, trachea, bronchus and lung], intestinal tract, liver, kidney, spleen, pancreas, brain and/or other tissues)

IAV identification workflows—Once samples have been collected, the workflow for virus identification may proceed in several ways (Figure 1). The ‘gold standard’ for IAV identification is virus culture in embryonated chicken eggs or mammalian cells, followed by a positive result in an HA assay, and subsequent verification of IAV in HA-positive samples by use of a more specific assay, such as RT-PCR analysis of genomic viral RNA (each of these methods is described in detail below). This workflow is highly sensitive and quickly produces a virus isolate ‘stock’ that can be used for further analysis and/or sent to WHO Collaborating Centre reference laboratories²¹ to enable vaccine strain selection and efficacy monitoring, or to aid in the identification of unknown virus subtypes. However, the ‘gold standard’ workflow is time-consuming and labor-intensive, and may not be feasible when rapid results are imperative or when hundreds or thousands of samples need to be assessed. Moreover, this approach may fail to identify IAV when viral material is present in samples but infectious virus is not, because no virus will be amplified in egg or cell cultures. An alternative approach to hasten sample evaluation and ensure IAV identification in the absence of infectious virus is to directly analyze sample material by using a rapid, specific and sensitive assay (i.e., RT-PCR). Both standard and real-time RT-PCR may be suitable for

this purpose^{22,23}, although real-time RT-PCR is more practical for large numbers of samples or when under a restrictive deadline because results are reported during the thermocycling procedure, without requiring subsequent gel electrophoresis and imaging steps. After the presence of IAV has been identified by using RT-PCR, virus cultures can then be prepared only from positive samples for use in downstream analyses. One disadvantage of this ‘fast’ workflow is that it may report false negative results in cases where viral RNA copy numbers are below the threshold for detection. Therefore, a third possible workflow – combining the ‘gold standard’ and ‘fast’ workflows – may be considered when both rapid and accurate results are needed. In this ‘combined’ workflow, samples are subjected to RT-PCR analysis directly to produce a preliminary set of positives within a short time period (i.e., usually the same day). In parallel, all samples are also subjected to virus culture, with follow-up HA assays and/or RT-PCR analyses to identify a secondary set of IAV-positive samples (virus culture may increase the identification of positive samples due to amplification of low initial virus amounts). Preliminary and secondary sets of positives are then compared to identify the complete set of positive samples. Selection of the appropriate IAV identification workflow should be determined according to project needs and laboratory resources.

Virus cultures—To prepare virus cultures from surveillance samples, aliquot(s) should be inoculated into embryonated chicken eggs or cell cultures, depending upon the sample’s origin. Most IAVs derived from avian species replicate efficiently in embryonated eggs (as originally demonstrated by Goodpasture et al.²⁴), and eggs are the preferred substrate for virus amplification from avian sources. Avian IAVs possess HA proteins that preferentially bind to terminal α 2-3-linked sialic acid moieties on cell surface molecules. These are abundant on the cells that line the allantoic cavity of embryonated eggs²⁵ (Figure 2A–B), and therefore, avian virus egg inoculations are directed to this region. Human IAV HA proteins prefer α 2-6 sialic acid linkages and replicate in the egg amniotic cavity (Figure 2A–B) because both α 2-3 and α 2-6 sialic acids are present in this compartment²⁵, although it should be noted that recent H3N2 viruses replicate poorly in this cavity^{26–28}.

Historically (i.e., before the availability of cell culture systems), human viruses were grown in eggs, and currently, most seasonal influenza vaccines are still produced this way. However, some human and swine IAVs replicate inefficiently in eggs, and passaging mammalian viruses in eggs can result in the acquisition of mutations that affect receptor binding specificity and/or antigenicity^{25,29,30}. As an alternative, IAVs can be amplified in mammalian cell cultures. Several primary and continuous mammalian cell types are suitable for IAV propagation, but the most frequently used is the Madin-Darby canine kidney (MDCK) cell line. MDCK cells were first described in 1966³¹ and were shown to support replication of some IAVs shortly thereafter³². Later studies indicated that addition of trypsin to MDCK cultures allowed for more efficient propagation and sensitive detection of IAVs from human specimens when compared to cultures lacking trypsin^{33–35}. Trypsin facilitates virus growth in MDCK cells by mediating cleavage of the virion surface glycoprotein precursor – HA0 – into HA1 and HA2 subunits^{36,37}, an event that is required for HA fusion with the endosomal membrane and release of genetic material into a newly infected cells³⁸. MDCK cells are relatively resistant to continuous culture with trypsin and express proteins bearing sialic acids with both α 2-3 and α 2-6 linkages at their surfaces²⁵, making them an

ideal cell type for propagation of IAVs that do not grow well in eggs. MDCK cells are usually permissive for mammalian IAVs, but are less so for viruses derived from avian species. Of note, a recent innovation in the isolation and propagation of current human influenza viruses is the generation of MDCK cells that are engineered to express increased levels of α 2,6-linked sialic acid receptors³⁹. These cells may enhance the isolation of human IAVs from clinical specimens and increase virus culture titers relative to standard MDCK cells, and can be used interchangeably with standard MDCK cells in the techniques described in this protocol.

HA assay—A widely used, inexpensive, rapid and standard screening method for the detection of IAVs is the hemagglutination (HA) assay (originally developed by Hirst⁴⁰ and Francis et al.⁴¹), which is based on the ability of the viral HA protein to bind to and ‘agglutinate’ red blood cells (RBCs). HA binding to RBCs is mediated by α 2,3- and/or α 2,6-linked sialic acid moieties at the RBC surface. HA assays are performed by mixing dilutions of virus-containing samples with a standard amount of RBCs in microtiter plates and observing agglutination patterns after a period of incubation. Agglutination causes RBCs to form a sheet that settles at the bottom of a well – giving a cloudy appearance – while the lack of agglutination results in RBC settling to the bottom of the well in the form of a point of cells (i.e., a ‘button’) or a circle of cells (i.e., a ‘halo’), surrounded by relatively translucent buffer.

The amount of IAV HA protein in surveillance samples is usually insufficient to yield positive results in the HA assay; however, this assay is effective for screening large numbers of virus cultures before performing additional – more specific – diagnostic analyses (i.e., RT-PCR). Importantly, several factors need to be considered when using this assay, including the following: (1) a positive HA assay indicates the presence of viral HA protein, but does not require infectious virus; (2) some IAVs do not agglutinate RBCs efficiently, even if their infectivity is high; (3) the origin of the RBCs (e.g., chicken, turkey, guinea pig, horse) can significantly affect the HA activity of an IAV, due to species-specific expression patterns of α 2,3- and α 2,6-linked sialic acid moieties on RBC surfaces⁴²; and (4) hemagglutinin proteins from other viruses (e.g., paramyxoviruses) can induce RBC agglutination, so a positive HA assay result should always be followed with a secondary assay that can specifically identify the IAV. Avian RBCs (e.g., turkey) are preferred for use in HA assays because they are nucleated, heavy, and settle quickly in microtiter plates; thus, negative activity can be clearly differentiated from positive, agglutinating activity. In contrast, lighter, non-nucleated mammalian RBCs (i.e., guinea pig and human RBCs) can form ‘halos’ upon settling, making the differentiation between positive and negative activity potentially less clear. Currently circulating human H3N2 viruses lack the ability to agglutinate chicken RBCs⁴³, and therefore, chicken RBCs should not be used for HA assays for these viruses. In addition, some human IAVs have been shown to agglutinate guinea pig RBCs with high efficiency⁴⁴. With these points in mind, the source of RBCs for use in HA assays should be carefully selected depending on the viruses that are expected to be present. When the HA assay is used to screen samples for the presence of IAVs, useful controls include cell culture medium or egg fluids without virus (negative control) and a sample known to contain an IAV with efficient agglutinating activity (positive control).

RT-PCR—For specific IAV identification (from original surveillance samples or after virus culture in embryonated chicken eggs or cells), it is preferable to use a method that will detect viruses irrespective of antigenic differences in the HA protein, especially when the HA subtype of the prospective virus is unknown. Although IAV genomic sequences vary widely across subtypes and species-specific virus groups, the ‘M’ gene segment is relatively highly conserved. Therefore, the presence of IAV can be evaluated by using either standard or real-time RT-PCR with primers that recognize the M gene of viruses of all subtypes. Real-time RT-PCR is favored because results can be obtained more rapidly; however, M gene sequences amplified by using standard RT-PCR can be evaluated by using gel electrophoresis with only a modest increase in the time necessary to complete the analysis, provided that the number of samples is relatively low. For quality control purposes, an egg or tissue culture sample known to contain an IAV should be included in all procedures required for the RT-PCR analysis (e.g., RNA extraction, cDNA synthesis, PCR amplification, and agarose gel electrophoresis), so that concerns about procedural efficacy can be excluded when no positive samples are identified. Importantly, care should be taken to avoid cross-contamination between the known positive sample and all other samples (e.g., by using aerosol-resistant micropipette tips and preparing the RT-PCR master mix in a clean location), and all RT-PCR reactions should include a ‘no-template negative’ control to ensure that the reagents used are not a source of contamination.

Other assays for IAV identification—HA assays and RT-PCR analysis are described in this protocol because they represent the most widely used and reliable methods for the identification of IAVs in surveillance samples and virus cultures. However, additional antigen-based methods also can be used for IAV identification. These include rapid, point-of-care assays that can both detect and distinguish between influenza A and influenza B viruses in about 30 minutes (e.g., Directigen EZ™ Flu A+B, Binax NOW® Influenza A & B), and immunofluorescence assays using influenza-specific antibodies. It is important to note that the successful use of rapid and immunofluorescence-based assays is highly dependent on the cellularity of the sample (i.e., more cells typically produce more accurate results)⁴⁵, and further that many reports indicate variable levels of sensitivity for these assays, which can lead to inadvertent false negative identifications (e.g., see^{45–49}). Moreover, immunofluorescence assays may require considerable sample manipulation, the use of sharp objects (i.e., glass slides) and the availability of specialized equipment (i.e., fluorescence microscopes), all of which provide significant obstacles when working under high containment conditions (i.e., in biosafety level 3 laboratories). Nonetheless, both rapid and immunofluorescence-based assays are used frequently by clinical diagnostic laboratories when assessing specimens derived from humans exhibiting influenza-like illness. As a final point, serology-based assays (e.g., hemagglutination inhibition^{41,50} and microneutralization⁵¹) can be used to retrospectively (and indirectly) identify IAV infection through the detection of IAV-specific antibodies in serum, but are not useful for the direct isolation and identification of viruses from clinical or research specimens. In sum, for the reasons just described, antigen- and serology-based assays are not considered further in this protocol. For additional information about antigen-based assays, readers are referred to^{15,16,52}; and for procedural overviews of these assays, readers are referred to⁵³.

Limitations of the Protocol

The procedures described in this protocol have been used extensively to isolate, propagate, and identify IAVs of all subtypes from all types of experimental and surveillance samples. The protocol is robust, and virus amplification is likely to be successful in most cases. However, the amplification procedure may be unsuccessful if: (1) the samples contain very low amounts of virus; (2) the samples are mishandled (e.g., not kept at 4°C during transport and/or not frozen within 48–72 h of receipt); (3) the samples are contaminated with other organisms (e.g., fecal material with bacterial contamination); or (4) in rare instances, the samples contain IAVs that do not grow efficiently in eggs or mammalian cells (e.g., IAVs from bats³). In addition, it must be emphasized that neither the HA assay nor RT-PCR analysis with M-gene-specific primers can differentiate between IAV subtypes. Therefore, if a positive identification of IAV has been made for a particular sample, then subsequent analyses must be performed to identify the specific subtype (e.g., by use of subtype-specific RT-PCR primer panels¹⁷, genome sequencing with universal primer sets⁵⁴, or subtype-specific antibody panels in hemagglutination inhibition assays⁵⁵). These additional analyses are outside the scope of the current protocol, and thus, will not be discussed further here.

Safety Considerations

Shipping Infectious Biological Materials—Frequently, surveillance or other IAV-containing samples must be shipped (i.e., transferred) between national and international sites. Government-issued import and export permits or licenses may be required for national and/or international transfer of infectious (or potentially infectious) substances. To remain compliant with national and international laws and regulations, personnel planning to import or export samples known or expected to contain IAV should: (1) contact the appropriate national agencies to identify the required permits and licenses; and (2) obtain the required permits and licenses prior to making or receiving any shipments. Only personnel trained and certified in the shipment of hazardous materials should prepare shipments of infectious substances, and only personnel trained in the handling of infectious substances should receive and unpack shipments.

Biosafety—All methods involving the manipulation of samples containing (or potentially containing) infectious IAVs must be performed in a class II biosafety cabinet in appropriate biosafety level 2 (BSL-2) or BSL-3 containment conditions, while using appropriate personal protective equipment (PPE). The proper containment level and PPE requirements should be identified based on the actual or potential IAVs that are (likely to be) present, before any procedures are performed. Generally, BSL-2 conditions are suitable for contemporary human IAV strains (H1N1 and H3N2). However, a higher containment level may be necessary if a strain poses a pertinent threat to human or agricultural animal populations. BSL-3 containment is required for work with highly pathogenic avian influenza viruses (e.g., H5N1); the recently emerged H7N9 viruses from China; any newly emerging human IAV for which limited knowledge is available; and historical pandemic viruses that have caused extremely high human mortality (e.g., 1918 IAV) or for which little or no immunity exists in the human population (e.g., H2N2 IAV). Personnel planning experiments with samples that are known or expected to contain IAV must consult with institutional committees and governmental agencies to ensure that all appropriate permits and licenses

are acquired prior to initiating any work. An excellent resource describing various types of biosafety containment and PPE is the ‘Biosafety in Microbiological and Biomedical Laboratories, 5th Edition’ (BMBL5), which is published by the United States Centers for Disease Control (CDC) and can be freely downloaded from the CDC website⁵⁶.

MATERIALS

Reagents

Biosafety

- 70% ethanol
- Disinfectant (e.g., Microchem Plus, 5%)

Sample collection

- Source of sample. See Introduction for information about the types of samples that can be used.

!CAUTION Experiments involving live animals must conform to local and national regulations. Legally effective informed consent may be required for experiments involving samples acquired from human subjects. Experiments involving human subjects must conform to local and national regulations.

- Phosphate-buffered saline (PBS), pH 7.2, autoclaved and stored at room temperature (20–25°C)
- Sterile glycerol, stored at room temperature
- Deionized water (dH₂O), autoclaved and stored at room temperature
- 10X Minimum Essential Medium (MEM) (+) Earle’s salts, (–) L-glutamine, (–) sodium bicarbonate (Gibco, cat. no. 11430-030); stored at 4°C for 2 months
- Bovine serum albumin (BSA) fraction V solution, 7.5% (Sigma-Aldrich, cat. no. A8412-100ml); stored at 4°C for 2 months or aliquoted, stored at –20°C, and thawed before use

▲CRITICAL STEP Some commercial sources of BSA fraction V impair TPCK-trypsin activity (unpublished data), which is required for IAV culture in cells. BSA from these sources should not be used for making media that will be used for sample collection or virus propagation (see below). We recommend Sigma-Aldrich BSA fraction V solution.

- Sodium bicarbonate solution, 7.5% (Gibco, cat. no. 25080-094); stored at 4°C for 2 months
- MEM amino acids, 50X (Gibco, cat. no. 11130-051); stored at 4°C for 2 months or aliquoted, stored at –20°C, and thawed before use
- MEM vitamin solution, 100X (Gibco, cat. no. 11120-052); aliquoted, stored at –20°C, and thawed before use

- L-glutamine, 200 mM (Gibco, cat. no. 25030-081); aliquoted, stored at -20°C , and thawed before use
- ‘Anti-Anti’ antibiotic-antimycotic mixture (10,000 units/ml of penicillin, 10,000 $\mu\text{g/ml}$ of streptomycin, and 25 $\mu\text{g/ml}$ of FungizoneTM), 100X (Gibco, cat. no. 15240-062); aliquoted, stored at -20°C , and thawed before use

Virus culture in eggs

- Fertilized (embryonated), pathogen-free chicken eggs, 10–12 days old
!CAUTION Experiments involving live animals must conform to local and national regulations.
- Egg sealant (e.g., paraffin wax, nail polish, or Elmer’sTM glue)
- PBS (prepare as described above)
- Anti-Anti (prepare as described above)
- Blood-agar plates (to check for bacterial contamination)

Virus culture in cells

- Madin-Darby canine kidney (MDCK) cells, (ATCC, cat. no. CCL 334)
- 0.25% trypsin-EDTA (Gibco, cat. no., 25200-056)
- PBS, autoclaved dH₂O, 10X MEM, BSA fraction V, sodium bicarbonate, MEM amino acids, MEM vitamin solution, L-glutamine, and ‘Anti-Anti’ (prepare each component as described above)
- Normal calf serum (NCS), heat-inactivated (Thermo Scientific, cat. no. SH30118.30); aliquoted, stored at -20°C , and thawed before use
- Tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (‘TPCK-trypsin’) (Worthington Biochemical Corporation, cat. no. TRTPCK)
▲CRITICAL STEP The addition of TPCK to trypsin preparations impairs the activity of contaminating chymotrypsin⁵⁷, which does not promote IAV infectivity but can cause damage to cell monolayers. We recommend using only TPCK-treated trypsin for IAV culture in MDCK cells.

HA assay

- Turkey red blood cells (RBCs), 10% (Rockland, cat. no. R408-0050)
- Guinea pig RBCs, 10% (Rockland, cat. no. R402-0050)
- PBS (prepare as described above)

RNA isolation

- Qiagen RNeasy mini RNA isolation column kit (Qiagen, cat. no. 74104)
- 2-mercaptoethanol

- 70% ethanol, molecular grade

Standard RT-PCR

- nuclease-free dH₂O
- dNTP mix, 10 mM (Life Technologies, cat. no. 18427013)
- RNasin Plus RNase inhibitor (Promega, cat. no. N2611)
- Superscript III one tube reverse transcriptase system (Invitrogen, cat. no. 12574-018)
- Forward and reverse oligonucleotide primers to the IAV M gene segment (as previously described in Annex 1-A of¹⁷; these primers have been validated for the universal detection of IAVs of all subtypes from human samples). They can be ordered from IDT, Inc.; diluted to 10 µM in dH₂O and stored at –20°C until use. The primer sequences are listed in the table below; “Y” refers to C or T; “N” refers to A, G, C, or T:

M30F2/08,	ATGAGYCTTYTAACCGAGGTCGAAACG
M264R3/08,	TGGACAAANCGTCTACGCTGCAG

▲CRITICAL STEP It should be noted that the primer set detailed above represents one of multiple validated primer sets described in¹⁷. Researchers with concerns about the appropriate primers to use in standard RT-PCR analysis should contact a WHO Collaborating Centre^{21,58} for additional guidance.

Real-time RT-PCR

- nuclease-free deionized H₂O
- dNTP mix, 10 mM (Life Technologies, cat. no. 18427013)
- 10X PCR buffer I with 15 mM MgCl₂ (Applied Biosystems, cat. no. 4379876)
- Random hexamer, 50 µM (Applied Biosystems, cat. no. 8080127)
- MuLV Reverse Transcriptase 50 U/µl (Applied Biosystems, cat. no. 8080018)
- Forward and reverse oligonucleotide primers (diluted to 10 µM) and fluorogenic probes (diluted to 5 µM) to the IAV M gene segment (as previously described in Annex 2-A of¹⁷; these primers have been validated for the universal detection of IAVs of all subtypes from human samples); ordered from IDT, Inc. and diluted in dH₂O. Prepare a ‘Primer/Probe Master Mix’ by mixing equal volumes of each; aliquot and store at –20°C until use. The sequence of each primer is given in the table below:

FLUAM-1F,	AAGACCAATCCTGTACCTCTGA
FLUAM-1P,	5'-(FAM)-TTTGTGTTACGCTACCGT-(TAMRA)-3'
FLUAM-1R,	CAAAGCGTCTACGCTGCAGTCC
FLUAM-2F,	CATTGGGATCTTGCACTTGATATT
FLUAM-2P,	5'-(FAM)-TGGATTCTTGATCGTCTTTTCTTCAAATGCA-(TAMRA)-3'

FLUAM-2R, AAACCGTATTTAAGGCGACGATAA

▲CRITICAL STEP It should be noted that the primer set detailed above represents one of multiple validated primer sets described in¹⁷. Researchers with concerns about the appropriate primers to use in real-time RT-PCR analysis should contact a WHO Collaborating Center^{21,58} for additional guidance.

- LightCycler® FastStart™ DNA Master HybProbe kit (Roche Applied Sciences, cat. no. 03 003 248 001)

Agarose gel electrophoresis

- 1 KB Plus DNA Ladder (Invitrogen, cat. no. 10787-018)
- 6X EZ-Vision One DNA loading dye, ethidium bromide-free (Amresco, cat. no. N472-KIT)
- Seakem ME agarose powder (Lonza, cat. no. 50014)
- dH₂O, autoclaved and stored at room temperature
- 10X TAE (Biorad, cat. no. 161-0773)

Equipment

Biosafety

- Class II biosafety cabinet fitted with an ultraviolet (UV) light
- Approved biosafety level 2 (BSL-2) or enhanced BSL-2 (BSL-2+) laboratory facilities for working with most mammalian and some avian IAVs
- Approved biosafety level 3 (BSL-3), enhanced BSL-3 (BSL-3+) or BSL-3 Agriculture (BSL-3-Ag) containment facilities for handling highly pathogenic avian IAVs

▲CRITICAL STEP Identify the proper BSL-2 or BSL-3 containment level based on the actual or potential IAV subtypes that are (likely to be) present in the sample(s), and ensure that all experiments conform to relevant institutional and governmental biosafety regulations.

- Personal protective equipment (PPE). For BSL-2, powder-free latex or nitrile gloves, laboratory coats, and eye protection. For BSL-3, scrubs, dedicated shoes, shoe covers, powder-free latex or nitrile gloves, water-proof Tyvek™ jumpsuits, water-proof Tyvek™ shrouds, powered air purifying respirators, water-proof Tyvek™ sleeve covers
- Biohazard trash bags
- Biohazard sharps containers
- Paper towels
- Spray bottles for 70% ethanol

- Dedicated container for disinfecting solid and liquid waste contaminated with virus
- Autoclave

Common equipment

- Micropipettes (1–1000 µl capacity)
- Pipet-Aid™
- Vortex mixer
- Electronic scientific or analytical balance
- Water bath, 37°C
- Refrigerated microcentrifuge (for 1.5 ml and 2 ml microtubes)
- Refrigerated table-top centrifuge (for 15 ml and 50 ml conical tubes)
- Freezers and refrigerators: –80°C, –20°C, 4°C
- Ice machine and ice buckets

Common consumables

- Sterile glass or plastic bottles (500 ml)
- Sterile polypropylene conical tubes, 15 ml (BD Falcon, cat. no. 352096) and 50 ml (BD Falcon, cat. no. 352070)
- Sterile screw-cap microtubes, 2 ml (Sarstedt, cat. no. 72.964)
- Snap-cap microtubes, 1.5 ml, autoclaved (Eppendorf, cat. no. 022363204)
- Sterile, aerosol-resistant micropipette tips (1–1000 µl capacity)
- Cotton-plugged, sterile serological pipettes (1–25 ml capacity)
- 0.2 µm syringe and bottle-top microfilters

Sample collection and processing

- Polyester fiber-tipped swabs (e.g., nylon or Dacron™) with plastic or wire shafts; for each swab sample collection scenario, use the largest swab size that maintains human or animal safety

▲CRITICAL STEP For improved sample collection, we suggest using ‘flocked swabs’ (Copan Diagnostics, Inc.), which have high absorbency, promote the collection of solid and semi-solid materials, and efficiently elute samples into transport medium. Flocked swabs have been reported to produce better clinical specimens for diagnosis⁵⁹; have outperformed standard, invasive nasal aspiration methods in collection of IAVs from humans^{60,61}; and have been found to yield higher virus amounts compared to rayon swabs⁶². Calcium alginate and cotton swabs are not recommended because residues present in these materials interfere

with PCR assays⁶³ (which will be performed downstream), and wooden shafts should be avoided because they may contain formaldehyde and/or other toxins.

- For tissue harvesting and processing: Tissue dissection tools (scissors, forceps), cleaned with soap and water and sterilized with 70% ethanol; a small brush for cleaning tools between specimen collections; 5-mm stainless steel homogenization beads, autoclaved (Qiagen, cat. no. 69989); homogenization bead dispenser (Qiagen, cat. no. 69965); TissueLyser II (Qiagen, cat. no. 85300)
- Ice packs, coolers and liquid nitrogen dry shipping containers for field collections

Virus culture in eggs

- Egg trays
- Egg candling light
- Rotating and static egg incubators (35°C, humidified)
- Egg piercing tool: 20-gauge, 1.5" catheter punched through a rubber stopper (for piercing egg air sacs; Figure 2C, **panel i**); an appropriately sized rubber stopper can be obtained from a 2-ml blood collection tube (e.g., Becton Dickinson, cat. no. 367844)
- 1-ml syringes with 27-gauge, 1" or 1.5" hypodermic or blunt-end needles (for inoculating eggs)
- 3-ml syringe with a 21-gauge, 1" hypodermic needle (for harvesting allantoic fluids)
- Sterile forceps

Virus culture in cells

- Tissue culture incubator (33–37°C, humidified, 5% CO₂)
- Straight neck polystyrene tissue culture flasks with vented caps, 25 cm² (BD Falcon, cat. no. 354484), 75 cm² (BD Falcon, cat. no. 353136), and 175 cm² (BD Falcon, cat. no. 353112)
- 6-, 12- or 24-well polystyrene tissue culture plates with lids (BD Falcon, cat. nos. 353046, 353043, or 353047, respectively)
- TC20 automated cell counter and plastic cell counting slides (Biorad) or a hemacytometer
- Cell culture microscope

HA assay

- Sterile gauze
- 96-well microtiter plates, U-bottom (Thermo Scientific, cat. no. 2205)

Standard RT-PCR

- PCR strip tubes, 0.2 ml (Eppendorf, cat. no. 951010022)
- Thermocycler

Real-time RT-PCR

- MicroAmp fast optical 96-well reaction plates, 0.1 ml (Applied Biosystems, cat. no. 4346906)
- Real-time thermocycler (e.g., 7900 HT Fast Real-Time PCR System, Applied Biosystems)
- Real-time RT-PCR analysis software (e.g., SDS Software, version 2.3, Applied Biosystems)

Agarose gel electrophoresis

- Microwave
- 500-ml, glass Erlenmeyer flask
- Agarose gel casting tray
- Electrophoresis chamber
- Power supply
- UV light box
- Imaging system for capturing images of agarose gels

Reagent Setup

- **Glycerol-saline transport medium ('GSTM') for collecting samples that will be inoculated into eggs.** Dilute 100X Anti-Anti at a ratio of 1:100 (v/v) with a 1:1 (v/v) solution of autoclaved PBS and sterile glycerol. Store at 4°C and use for up to 1 month.

▲**CRITICAL STEP** Use this solution only for collecting samples that will be inoculated into eggs. GSTM imparts longer term stability to viruses than does MEM-BSA (see details about MEM-BSA preparation below), particularly in conditions where samples cannot be placed immediately on ice¹⁶. However, the high glycerol concentration in GSTM is not suitable for mammalian cell cultures⁶⁴ and should not be used to collect samples that will be inoculated into MDCK cells.

- **PBS-AA (PBS containing 1X Anti-Anti) for diluting samples that will be inoculated into eggs.** Dilute 100X Anti-Anti at a ratio of 1:100 (v/v) in autoclaved PBS. Store at 4°C and use for up to 1 month.
- **10–12-day-old embryonated chicken eggs.** Prior to use, “candle” eggs by placing them in front of a light source. Ensure that the egg is fertilized and viable, and that the shell is not damaged. Using a pencil or an ethanol-resistant

marker, designate a position just above the interface between the air sac and the allantoic sac that is free of veins (this position will be used for inoculation) (Figure 2B).

- **MEM-BSA for collecting samples that will be inoculated into MDCK cells and for virus culture in MDCK cells.** First, prepare and store a 2X MEM-BSA solution as follows: In a 500-ml bottle, mix the following components (total solution volume, 500 ml): 270 ml of dH₂O; 100 ml of 10X MEM (+) Earle's salts, (–) L-glutamine, (–) sodium bicarbonate (final concentration: 2X (v/v)); 40 ml of 7.5% BSA fraction V solution (final concentration: 0.6% (v/v)); 30 ml of 7.5% sodium bicarbonate solution (final concentration: 0.45% (v/v)); 20 ml of 50X MEM amino acids solution (final concentration: 2X (v/v)); 10 ml of 100X MEM vitamins solution (final concentration: 2X (v/v)); and 20 ml of 200 mM L-glutamine (final concentration: 16 mM (v/v)). Filter the mixture using a bottle-top 0.2-µm filter, and then add 10 ml of 100X Anti-Anti (final concentration: 2X (v/v); or 400 units/ml penicillin, 400 µg/ml streptomycin, and 1 µg/ml of Fungizone). Prepare media in advance, store at 4°C, and use it for up to one month. On the day of the experiment, prepare the required amount of MEM-BSA by mixing 2X MEM-BSA at a 1:1 ratio with dH₂O.
- **MEM-NCS (MDCK cell growth medium, containing 10% NCS).** In a 500-ml bottle, mix the following components (total solution volume, 500 ml): 380 ml of dH₂O; 50 ml of 10X MEM (+) Earle's salts, (–) L-glutamine, (–) sodium bicarbonate (final concentration: 1X (v/v)); 25 ml NCS (final concentration: 5% (v/v)); 15 ml of 7.5% sodium bicarbonate solution (final concentration: 0.23% (v/v)); 10 ml of 50X MEM amino acids solution (final concentration: 0.5X (v/v)); 5 ml of 100X MEM vitamins solution (final concentration: 1X (v/v)); and 10 ml of 200 mM L-glutamine (final concentration: 8 mM (v/v)). Filter the mixture using a bottle-top 0.2-µm filter, and then add 5 ml of 100X Anti-Anti (final concentration: 1X (v/v); or 200 units/ml penicillin, 200 µg/ml streptomycin, and 0.5 µg/ml of Fungizone). Prepare media in advance, store at 4°C, and use it for up to one month.
- **TPCK-trypsin stock solution (1 mg/ml in dH₂O) for use in virus growth medium (see below).** Prepare a stock solution in advance and store 1-ml aliquots at –20°C; thaw individual aliquots at the time of use. For the stock solution, dissolve 0.1 g of TPCK-trypsin into 100 ml of autoclaved dH₂O and vortex vigorously to mix. Filter the solution by passaging through a 0.2-µm syringe microfilter before aliquoting into microtubes.

▲CRITICAL STEP Prior to use for virus propagation in MDCK cells, titrate the maximum TPCK-trypsin concentration (from a thawed aliquot) that can be tolerated by MDCK cells (i.e., the highest concentration at which minimal or no detrimental effects are observed³⁴), and use this concentration for all IAV propagation assays in the same cell stock. Tolerable concentrations vary between TPCK-trypsin stock solutions and MDCK cell stocks, so titration should be performed every time a new stock solution is generated or when using MDCK

cells from an alternate source. In our experience, tolerable TPCK-trypsin levels range from 0.5–1 µg/ml final concentration in MEM-BSA.

- **MEM-BSA-TPCK (virus growth medium).** Prepare MEM-BSA as described above and add TPCK-trypsin to a final concentration of 0.5–1 µg/ml.
- **MDCK cell culture.** To establish MDCK cell cultures, thaw a low passage (< 10) vial of liquid nitrogen-preserved cells (containing 10% DMSO), dilute with 10 ml of cold MEM-NCS, centrifuge ($200 \times g$, 2 minutes at room temperature), and resuspend the pellet in 20 ml cold MEM-NCS. Seed a 75-cm² flask with the entire volume of resuspended cells and incubate at 37°C. Roughly 48 h later, split the cells at a 1:10 dilution and continue to do so every 2–3 days.
- **Turkey RBCs, 0.5%, for HA assays.** Filter 10% RBCs through sterile gauze, pellet cells by centrifugation ($200 \times g$, 10 minutes at 4°C) and remove plasma, Alsever's solution, and buffy coat. Wash RBCs three times with excess, cold PBS, and then resuspend to 0.5% in cold PBS for use.
- **Guinea pig RBCs, 0.75%, for HA assays.** Prepare as just described for turkey RBCs, except resuspend to 0.75% in cold PBS for use.
- **1X TAE for agarose gel electrophoresis.** Mix 10X TAE with dH₂O at a 1:10 (v/v) ratio to the desired volume.
- **1% agarose gel.** Mix 1 g of agarose with 10 ml of 10X TAE and dH₂O to 100 ml in a 500-ml glass Erlenmeyer flask. Microwave on high for 1–2 minutes or until the agarose is completely dissolved. Cool the agarose solution slightly, pour into an agarose gel cast, and allow the gel to set.

Equipment Setup

- **Class II biosafety cabinet.** Ensure the biosafety cabinet (BSC) is air-flow certified and fully decontaminated with 70% ethanol and UV light prior to use. A dedicated plastic container containing an appropriate disinfectant should be placed inside the BSC prior to beginning any work with infectious agents for decontaminating solid and liquid waste containing viruses. A biohazard sharps collection container also should be placed inside the BSC whenever sharp objects will be used in conjunction with IAVs.
- **Centrifuges.** All centrifugation steps must be performed at 4°C; therefore, cool all centrifuges prior to use. Use only rotors with aerosol-tight lids for centrifuging samples containing (or potentially containing) IAVs.
- **Electrophoresis chamber.** Place the casted 1% agarose gel into the electrophoresis chamber and cover with 1X Tris-acetate-ethylenediaminetetraacetic acid (TAE).
- **Incubators.** Set incubator(s) to the appropriate temperatures for cell and virus cultures, and supply a tray of water to provide humidity. For cell culture incubators, infuse with an atmosphere of 5% CO₂.

- **Autoclave.** For decontamination of laboratory wastes (e.g., pipettes, tissue culture vessels, used tubes) and discarded PPE, program autoclaves to cycle for at least 1 hour at 125°C and 20 pounds of pressure.

!CAUTION If animal or egg remains are frozen prior to autoclaving, ensure that they are completely thawed before initiating decontamination by autoclaving.

PROCEDURE

Part 1: Sample collection and preparation for virus amplification

1 If samples are swabs or liquid, follow option A for collection and preparation. If samples are tissues, follow option B

A) Swab and liquid sample collection and preparation

- Prepare sample collection tubes for swab samples by dispensing 1–3 ml of the appropriate transport medium into sterile plastic tubes. For swab samples that will be inoculated into MDCK cell cultures, use MEM-BSA; for those that will be inoculated into eggs, use either MEM-BSA or GSTM. Use sterile plastic collection tubes without transport medium for liquid sample collections. If feasible, chill all sample collection tubes and transport buffers to 0–4°C prior to use, as this temperature has been shown to preserve viral RNA, hemagglutinating activity and virus infectivity^{65–67}.

■PAUSE POINT Tubes can be prepared in advance and stored at 4°C for up to 1 week before use.

- Fully saturate dry polyester fiber-tipped swabs by inserting into the target mucous membrane region, pausing for a few seconds, and then removing while gently rotating and wiping as much surface area as possible. For nasal or nasopharyngeal surfaces, obtain specimens from both nostrils using the same swab. Obtain fecal samples by coating a swab tip in freshly excreted, wet fecal material. Collect liquid samples directly from the source without using a swab.
- Immediately place saturated swabs into plastic collection tubes (prepared as described in **substep i**); break off the swabs' shafts, leaving the heads immersed, close tubes, and place them on ice. For non-swab liquid samples, immediately close the collection tube and place on ice.
- Transfer samples directly to the laboratory, or if in the field, to a liquid nitrogen dry shipping container for transport. Once in the laboratory, store samples that previously were not frozen at 0–4°C if processing and inoculation can be performed within 48–72 h; otherwise, freeze samples at –80°C.

■PAUSE POINT Do not store samples longer than 72 h at 4°C to avoid loss of viability^{65–67}. If frozen, samples can be stored at –80°C for a minimum of several months. However, some loss of viability will occur after the initial freeze, and further viability reductions are increasingly likely as the length of storage time is increased⁶⁸.

▲CRITICAL STEP Avoid repeated freeze-thaw cycles, as each cycle reduces IAV viability⁶⁸. Consider dividing samples into aliquots. Samples should not be frozen or shipped on dry ice unless they are sealed, taped, and double-bagged because CO₂ exposure can rapidly reduce IAV viability/infectivity⁶⁹.

- v. To prepare samples for inoculation, thaw rapidly in a 37°C water bath or incubator (if required). Vortex vigorously and allow debris to settle for 30 minutes while holding on ice. After debris has settled, advance to virus amplification steps (**Part 2**) and/or RT-PCR analysis (**Part 3**); return any remaining sample to –80°C. Do not filter samples.

▲CRITICAL STEP It is important to avoid using microfilters to remove bacteria and fungi from samples (even fecal samples), as microfilters frequently also remove viruses. To prevent bacterial and fungal outgrowth in inoculated cultures, include antibiotics and antimycotics in all transport, inoculation, and culture media, as described in ‘Reagent Setup’.

B) Tissue sample preparation and processing

- i. Prepare sterile sample collection tubes without transport medium for each individual organ sample. If feasible, chill sample collection tubes on ice prior to sample collection.
- ii. Dissect tissues from deceased animals as soon as possible following their expiration. For larger organs (e.g., ferret lung), collect multiple tissue samples from several unique locations to increase the likelihood of virus isolation. Immediately place the dissected tissue samples on ice.
- iii. Transfer tissue samples to the laboratory or to a liquid nitrogen dry shipping container for transport to the laboratory. Once in the laboratory, immediately process tissue samples that were not frozen; and if this is not possible, freeze (without transport medium) at –80°C.

■PAUSE POINT Tissue samples can be stored at –80°C for a minimum of several months. However, some loss of virus viability will occur after the initial freeze, and further viability reductions are increasingly likely as the length of storage time is increased⁶⁸.

- iv. Prepare tissues for downstream analysis by homogenizing them. Thaw frozen tissues on ice and weigh using a scientific or analytical balance (the balance should be placed inside the biosafety cabinet). Transfer a section of each thawed tissue (0.5 g) to a 1.5-ml microtube or a 2-ml cryovial, along with one sterile stainless steel 5-mm homogenization bead and 1 ml of PBS-AA (for egg inoculations) or MEM-BSA (for MDCK cell inoculations).
- v. Secure all tube caps, load the tubes into a TissueLyser II rack, and attach the rack to the TissueLyser II clamps; then run one homogenization cycle (30 Hz oscillation frequency for 3 minutes).

- vi. Remove the tubes from the homogenization rack and visually check for complete tissue disruption. If tissue pieces remain, repeat the homogenization cycle until the tissues are completely homogenized. Prevent sample heating by resting the tubes on ice between homogenization cycles.

?TROUBLESHOOTING

- vii. Centrifuge completely homogenized tissues at 4°C for 10 minutes ($20,000 \times g$) to pellet debris, and transfer the cleared tissue supernatants (containing released virus) into new sterile microtubes or cryovials. As described in **subpart v**, do not microfilter the supernatants.
- viii. Place the tissue supernatants on ice and proceed to the virus amplification (**Part 2**) and/or RT-PCR analysis (**Part 3**). Following inoculation, return any remaining tissue supernatants to -80°C .

Part 2: Virus amplification in and harvest from embryonated chicken eggs or MDCK cell cultures

2 Perform virus amplification and harvest in embryonated chicken eggs (option A) or MDCK cell culture (option B). Further information about which system to use for particular samples is given in the Introduction

A) Embryonated eggs

- i. Determine the number of eggs required for the samples to be tested. For initial virus recovery from human or other surveillance or experimental samples, use 3 eggs per sample to be assayed. When very low rates of positive samples are anticipated from a large number of specimens, consider pooling samples prior to egg inoculation. If a pooled sample is identified as positive for IAV, individual samples that make up the pool may be tested subsequently. To prepare stocks of known viruses, inoculate multiple eggs with the same sample.
- ii. Spray the required number of candled and marked eggs (in an egg carton tray) with 70% ethanol to decontaminate surfaces, and then place the tray inside of a biosafety cabinet (see 'Reagent Setup' for additional details on egg candling).
- iii. Using a homemade egg piercing tool, made from a 20-gauge catheter pushed through a rubber stopper, gently pierce the egg shell at the location marked during candling (Figure 2A–B and 2C, **panels i and ii**). Avoid cracking the egg shell.

!CAUTION To avoid unnecessary sharps use in BSL-3 containment, perform all egg piercings in BSL-2 prior to entering BSL-3 containment to perform inoculations.

- iv. For each inoculation, draw up 100 μl of sample and 100 μl of PBS-AA into a 1-ml syringe with a 27-gauge, 1" needle (for inoculation of the allantoic cavity) or a 1.5" needle (for inoculation of both the allantoic and amniotic cavities).
- v. For inoculation of both the allantoic and amniotic cavities, insert the inoculation needle (1.5") at a 45° angle into the pierced region of the egg, gently push

through the allantoic sac and into the amniotic sac, and inject 100 μ l (one half) of the inoculum (Figure 2C, **panel iii**). Pause to allow the inoculum to flow completely from the syringe, and then draw the needle back into the allantoic region and expel the remainder of the inoculum. For inoculation of the allantoic cavity only, insert the inoculation needle (1") directly into the allantoic sac and expel the contents.

!CAUTION To prevent inadvertent exposures, handle sharps (i.e., needles) with extreme caution when manipulating samples that contain (or potentially contain) infectious IAVs. In particular, we recommend using blunt-ended (not hypodermic) needles for all allantoic cavity inoculations, and to avoid performing amniotic cavity inoculations – thereby eliminating the need for hypodermic needles – whenever possible.

?TROUBLESHOOTING

- vi.** Remove the syringe and safely discard it into a sharps container within the biosafety cabinet.
- vii.** Seal the piercing using a drop of melted wax, glue, or nail polish.
- viii.** When all eggs have been inoculated and sealed, decontaminate egg surfaces by spraying with 70% ethanol and then remove them from the biosafety cabinet.
- ix.** Incubate eggs at 35°C in a humidified, static incubator for 2–3 days.
- x.** At the completion of the incubation period, prepare eggs for fluid harvesting by placing them on ice (at 0–4°C) for 1h.

■ **PAUSE POINT** Eggs can be kept at 0–4°C overnight.

▲ **CRITICAL STEP** The 0–4°C treatment is required to euthanize the embryo and to prevent bleeding during fluid harvesting procedures. Because influenza viruses adsorb to RBCs, the presence of RBCs in egg fluids may reduce the amount of recoverable virus, as heavy debris (including RBCs) will be removed by centrifugation in a later step.

- xi.** Remove eggs from the 0–4°C treatment, decontaminate surfaces by spraying with 70% ethanol, and place in a biosafety cabinet.
- xii.** Use sterile forceps to crack the egg in the region of the air sac (Figure 2D, **panel i**). Remove cracked pieces of shell and dispose of them in a container of disinfectant.

!CAUTION Avoid removing pieces of eggshell below the top of the allantoic sac to prevent premature disruption and/or allantoic fluid spills, which could result in a major biohazard.

▲ **CRITICAL STEP** Decontaminate forceps between egg harvests by using 70% ethanol or an alternative disinfectant.

- xiii.** To obtain the allantoic fluid, use sterile forceps, a micropipette tip, or a serological pipette to gently detach the allantoic membrane from the shell and

pull it toward the center of the egg, while avoiding breaking blood vessels or the yolk (Figure 2D, **panel ii**). Fluids will accumulate in the region between the shell and the allantoic membrane. Harvest these fluids into a conical tube by using a micropipette (Figure 2D, **panel iii**) or a serological pipette (not shown). Typically, 5–10 ml of allantoic fluid can be harvested from each egg.

▲CRITICAL STEP Because the presence of blood or yolk may affect the resultant virus titer or the efficacy of downstream assays, avoid harvesting egg fluids mixed with these substances.

▲CRITICAL STEP If using forceps, decontaminate between egg harvests by using 70% ethanol or another appropriate disinfectant.

?TROUBLESHOOTING

- xiv.** To harvest amniotic fluid, locate the amniotic sac by slowly inverting the egg over a container of disinfectant. Harvest the amniotic fluid by using a 3-ml syringe with a 21-gauge, 1" needle.

!CAUTION To avoid the unnecessary risk of a biohazard spill, do not perform this step if or when highly pathogenic avian IAVs may be present. Such viruses typically replicate well in the allantoic tissues, so amniotic sac inoculation and fluid collection is unnecessary.

- xv.** If desired, check for the presence of bacterial contamination in egg cultures. To do this, spread egg fluids onto blood-agar plates, incubate plates at 37°C for 24 hours, and observe bacterial growth relative to a negative control (e.g., fluid from a fresh, non-inoculated egg). Alternatively, inoculate tubes of fresh MEM (containing serum and lacking antibiotics/antimycotics) with egg fluids, incubate at 37°C, and examine the tubes for turbidity and pH change over a 24–72 hour period, relative to a negative control. Discard contaminated cultures and do not use them in downstream analyses.
- xvi.** Discard egg remains by placing them in a biohazard bag inside the biosafety cabinet, and then autoclaving and incinerating them.
- xvii.** If necessary, perform an HA assay or RT-PCR (standard or real-time) analysis to determine or verify the presence of IAV as described in **Part 3**. Depending on the results, process the egg fluids as described in **Box 1**.

■PAUSE POINT Fluids from inoculated eggs can be stored for up to 72 h at 4°C until HA or RT-PCR assays are completed.

B) MDCK cells

- i.** Grow up an appropriate number of MDCK cells. For initial virus recovery from human or other surveillance or experimental samples, use one 25-cm² vented tissue culture flask of MDCK cells for each sample to be assayed. Alternatively, individual wells of tissue culture plates (6-, 12- or 24-well) may be used. Use larger vented flask(s) of cells (e.g., 175 cm²) when preparing larger stocks of known viruses.

- ii. One day before inoculation, prepare MDCK cells by splitting actively growing, sub-confluent cultures and seeding inoculation cultures. To split cells, wash cells twice with PBS and incubate with 0.25% trypsin-EDTA (2 ml per 175-cm² flask, 10–20 minutes at 37°C) to create a single cell suspension. Neutralize the trypsin-EDTA by adding a 4-fold volume of MEM-NCS, and briefly vortex the mixture to create a homogenous solution.
- iii. Determine the cell number using a hemacytometer or a TC20 automated cell counter and seed cells in MEM-NCS at a sufficient number to produce 80%–90% confluence within 24 h (i.e., the time of inoculation). The precise number of cells will differ based on the passage number, medium freshness, and other experimental variables. Incubate the cells at 37°C in a humidified atmosphere of 5% CO₂ for 16–24 h.

!TROUBLESHOOTING

- iv. On the day of inoculation, remove the MDCK growth medium (MEM-NCS) and wash the cells 2–3 times with PBS, leaving the final wash on the cells until just before the inoculation. Cells also can be washed with MEM-BSA.

▲CRITICAL STEP This step is necessary to remove traces of serum and other inhibitors of TPCK- trypsin activity and IAV infectivity³⁴.
- v. Prepare virus growth medium (MEM-BSA-TPCK) for use in all inoculation and incubation procedures. See ‘Reagent Setup’ for additional details.
- vi. Dilute inoculation samples 1:100–1:1000 (v/v) in MEM-BSA-TPCK. Prepare a volume that covers cells (e.g., 300 µl in a 25-cm² flask or 2 ml in a 175-cm² flask), and vortex the dilution for 10 seconds to mix.
- vii. Remove the final wash from the cells (**from substep iv**) and cover with the inoculum (**from substep vi**). Incubate cells with inoculum for 1 h at 37°C. During the incubation period, manually rock or tap the flasks every 10 minutes to evenly distribute the inoculum across the monolayer and to avoid cell drying in the flasks’ central regions.
- viii. At the completion of the incubation period, remove the inoculum and wash the cells once with PBS. Cover the inoculated, washed cells with MEM-BSA-TPCK, in a volume suitable for MDCK cell culture over several days (e.g., 4 ml in a 25-cm² flask or 25 ml in a 175-cm² flask).
- ix. Incubate inoculated cells in a 33–37°C, humidified tissue culture incubator with an atmosphere of 5% CO₂ for 2–5 days, proceeding to the next step after 1 day.

▲CRITICAL STEP Most mammalian viruses will grow well at 35°C, so this is an appropriate temperature for initial isolation experiments with viruses whose properties are unknown. However, some viruses exhibit optimal growth at other temperatures (e.g., 37°C and 33°C), and in cases where viral replication levels are modest, alternative incubation temperatures should be considered. Do not perform medium changes during the incubation period, as this will remove most of the viruses that are present.

- x.** Monitor MDCK cultures microscopically on a daily basis to observe cytopathic effects (CPE). IAV-induced CPE include visible cell rounding and detachment from the growth surface (Figure 3); 2–3 days of virus amplification is usually sufficient to produce this effect in the majority of cells in a culture. If CPE is observed within 5 days, proceed to **substep xi**, otherwise proceed to **substep xii**.
- xi.** When 90% of cells in a given culture have detached from the flask, transfer the culture medium into a conical tube (samples derived from the same source can be pooled) and centrifuge to pellet the cellular debris ($3,200 \times g$, 4°C, 10 minutes). Aliquot the cleared supernatant into cryovials (0.5–1 ml per aliquot). Retain aliquots for HA assay and/or RT-PCR analysis, if required, and freeze the remaining aliquots at –80°C.

▲CRITICAL STEP Abundant CPE will induce moderate turbidity in the culture medium, although medium should remain semi-transparent. If excess turbidity (i.e., opaque medium) is visually observed; or if small, uniform granules (i.e., bacteria) or clumps or mats (i.e., yeast or fungi) are microscopically observed, the culture should be discarded due to contamination.

▲CRITICAL STEP Do not freeze IAV stocks at –20°C, as the virus is highly unstable at this temperature⁶⁸.

- xii.** If CPE are not observed after 5 days of MDCK cell culture incubation, passage an aliquot of culture medium from the inoculated cells onto a fresh flask of MDCK cells. A total of three passages (including the original inoculation) without any observed CPE is sufficient to classify a sample as ‘negative’ for IAV amplification in cell culture.

?TROUBLESHOOTING

- xiii.** At the end of all cell culture experiments, decontaminate flasks and other contaminated plastics by soaking them in disinfectant; dispose of them in biohazard waste, and then autoclave and/or incinerate them.
- xiv.** To determine whether MDCK supernatants contain IAV, proceed to **Part 3** to perform an HA assay or RT-PCR (standard or real-time) analysis

▲CRITICAL STEP RT-PCR is required only for surveillance samples, in which the presence of IAV is unknown; observation of CPE in cultures inoculated with a known IAV is sufficient to classify these cultures as IAV-positive.

■PAUSE POINT MDCK culture supernatants can be stored for up to 72 h at 4°C until HA or RT-PCR assays are completed.

Part 3: Virus Detection by use of HA Assay, Standard RT-PCR, or Real-Time RT-PCR

3 If appropriate, the presence of IAV in egg or MDCK cultures can be determined by using an HA assay (option A), standard RT-PCR (option B) or real-time RT-PCR (option C)

A) HA Assay

- i. Prepare turkey or guinea pig RBCs (see 'Reagent Setup').
- ii. Add 100 µl of undiluted allantoic fluid or MDCK culture medium to the 'first' well (upper left) of a 96-well U-bottomed microtiter plate and perform serial 1:2 dilutions (50 µl of egg fluid or cell culture medium in an equal volume of PBS) in the eleven adjacent wells (from left to right, 2^0 – 2^{-11} dilutions). Discard the excess 50 µl from the final dilution well. Repeat (in empty rows) for all samples to be tested.
- iii. Add 50 µl of washed RBCs (turkey, 0.5%; guinea pig, 0.75%) to each well of each serial dilution series and tap the plate to mix the well contents.
- iv. Incubate for 30 minutes (turkey RBCs) or 1 hour (guinea pig RBCs) at room temperature (20–25°C) without disturbing the plate.
- v. Observe all wells for agglutination activity, which is characterized by uniform RBC distribution throughout the well (i.e., a cloudy appearance; Figure 4A and B). Alternatively, in agglutination-negative samples, RBCs will settle to the bottom of the well and form a 'button' (turkey RBCs; Figure 4A and B) or a 'halo' (guinea pig [or human] RBCs; Figure 4A), surrounded by relatively translucent buffer. Samples that genuinely contain IAVs typically exhibit positive HA activity over a range of dilutions (Figure 4B).

■TROUBLESHOOTING

B) Standard RT-PCR analysis

- i. Harvest a 200 µl aliquot from surveillance samples, HA-positive egg fluids, or MDCK culture medium and mix with an equal volume of Qiagen RLT lysis buffer (from the Qiagen RNeasy mini RNA isolation kit) and 4 µl of 2-mercaptoethanol (1% of the total volume).
- ii. Add 400 µl of 70% ethanol and incubate the sample at room temperature (20–25°C) for 10 minutes. At this point, all infectious IAVs are neutralized, and the sample can be manipulated in BSL-2 containment, provided that all appropriate procedures for removing samples from BSL-3 containment have been observed.
- iii. Advance through the remainder of the Qiagen RNeasy mini kit protocol according to the manufacturer's instructions. Briefly, pass the RLT lysate over an RNeasy spin column, wash column-bound RNA once with buffer RW1 and then twice with buffer RPE; then elute the RNA into 50 µl of RNase-free dH₂O.

■PAUSE POINT Eluted RNA can be frozen (–20° to –80°C) indefinitely.

- iv. Set up a one-tube cDNA synthesis (i.e, reverse transcriptase) and PCR amplification reaction by using the Superscript III reverse transcriptase system and IAV M gene-specific primers (M30F2/08 and M264R3/08), according to the manufacturer's instructions. Use 0.2-ml PCR strip tubes to carry out the reaction. For each reaction, use 1–5 µl of template RNA, 1 µl of each primer, and include 1 µl of RNasin RNase inhibitor. Carry out the reaction in a thermocycler, as described in the following program for IAV M-gene specific cDNA synthesis and standard RT-PCR:

Cycle	cDNA synthesis	Denaturation	Annealing	Extension	Hold
1	50°C for 30 minutes				
2		94°C for 2 minutes			
3–47		94°C for 15 seconds	50°C for 30 seconds	68°C for 1 minute	
48				1 cycle: 68°C for 5 minutes.	
49					4°C for up to 1 week

▲CRITICAL STEP To avoid false positive results due to carry-over from previous reactions, it is essential to practice precautionary measures. Assay setup and analysis must be performed in dedicated spaces with dedicated equipment, and clean gloves must be used for setting up all assays. Equipment and workspaces should be decontaminated after each use, and if possible, thermocyclers should be exposed to UV light before use.

■PAUSE POINT Hold completed RT-PCR reactions at 4°C for up to 1 week until the presence of an M-gene amplification product can be evaluated by use of agarose gel electrophoresis.

- v. To determine whether products were amplified in the RT-PCR reaction, prepare a 1% agarose gel (see 'Reagent Setup') and submerge it in 1X TAE.
- vi. Dilute RT-PCR reactions by adding 10 µl of 6X DNA EZ Vision DNA loading dye to each 50 µl reaction.
- vii. Run 10–20 µl of each reaction on the 1% agarose gel.
- viii. By using a UV light box, determine whether a band consistent with the size of the M gene amplicon (244 base pairs) is present. No amplification should be observed in negative controls. Image the gel using a gel imaging system.

?TROUBLESHOOTING

C) Real-time RT-PCR

- i. i. Extract RNA as described for standard RT-PCR analysis (**Step 3, Option B, substeps i-iii**).
- ii. ii. Set up a cDNA synthesis (reverse transcriptase) reaction as described below:

Reagent	Volume (µl)	Final Concentration
10X PCR buffer I with 15 mM MgCl ₂	2	1X, 1.5 mM MgCl ₂
25 mM MgCl ₂	2.8	3.5 mM
dNTPs (2.5 mM)	8	1 mM
Random hexamer 50 µM	1	2.5 µM
RNase inhibitor (40 U/µl)	1	40 U
Reverse transcriptase (50 U/µl)	1	50 U
Extracted RNA	4.2	varied
Total	20	

Vortex to mix, incubate at room temperature for 10 minutes, and then at 42°C for at least 15 minutes.

- iii. Deactivate the reverse transcriptase by incubating at 95°C for 5 minutes, and then chill the reactions on ice.
- iv. Set up the real-time PCR reaction in a MicroAmp fast optical 96-well reaction plate as described in the table below. Note that the Primer/Probe Master Mix contains primer/probe sets against two highly conserved regions of the IAV M gene, and that the FastStart DNA Master HybProbe mix used is from the Roche LightCycler - FastStart DNA Master HybProbe kit.

Reagent	Volume (µl)	Final Concentration
dH ₂ O	7.6	Not applicable
25 mM MgCl ₂	2.4	3 mM
Primer/Probe Master Mix (see 'Materials')	3	0.75 µM primer 0.375 µM probe
10X LightCycler® FastStart DNA Master HybProbe	2	1X
cDNA from step ii	5	varied
Total	20	

- v. Perform the IAV M-gene specific real-time RT-PCR in a real-time thermocycler as described below:

Cycle	Activation	Denaturation	Annealing	Elongation	Cooling
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1	95°C for 10 minutes			
2–51	95°C for 10 seconds	56°C for 15 seconds	72°C for 10 seconds	
53				40°C for 30 seconds

- vi. When the reaction is complete, view the amplification plots according to the real-time apparatus manufacturer's instructions. Ensure that the threshold is set just above the background signal and real-time RT-PCR.

?TROUBLESHOOTING

Box 1: Further analysis of egg fluids

If you performed virus amplification and harvest in chicken eggs, you may need to proceed with one of the following options depending on the results of the HA assay and/or RT-PCR analysis. If HA activity or a positive RT-PCR result is obtained, follow option A. If samples are negative for HA activity or a negative RT-PCR result is obtained, follow option B.

A) If HA activity or a positive RT-PCR result is obtained

- i. Pool all positive egg fluids from the same source into a 50-ml conical tube.
- ii. Centrifuge to remove debris (3,200 × g, 4°C, 10 minutes).
- iii. Aliquot cleared supernatants into cryovials (0.5–1 ml per aliquot).
- iv. Freeze aliquots at –80°C.

▲CRITICAL STEP Do not freeze IAV stocks at –20°C, as the virus is highly unstable at this temperature⁶⁸.

B) For samples that are negative by HA and/or RT-PCR assays

- i. Passage the harvested egg fluids from the initial infection into fresh embryonated chicken eggs to amplify any existing virus (as described in **Step 2, Option A, Substeps i–xvii**).
- ii. If egg fluids remain HA- and/or RT-PCR-negative after the second passage, the sample is considered 'negative' for IAV amplification in eggs.

?TROUBLESHOOTING

TIMING

Part 1: Sample collection and preparation for virus amplification

Step 1, Option A: Swab and liquid sample collection and preparation

- **Substeps i–iv** (swab and liquid surveillance sample collections), 1 h to multiple days, depending on the number and type of samples
- **Substep v** (swab and liquid surveillance sample processing), < 1 h

Step 1, Option B.: Tissue sample preparation and processing

- **Substeps i–iii** (animal tissue collection), 1–6 h, depending on the number of animals
- **Substeps iv–viii** (animal tissue homogenization), 1–2 h

Part 2: Virus amplification and harvest in embryonated chicken eggs or MDCK cell cultures**Step 2, Option A: Embryonated chicken egg inoculation, fluid harvest and fluid processing**

- **Substeps i–ix** (inoculation), 1 h followed by 2–3 days incubation
- **Substep x** (egg chilling), 1–16 h
- **Substeps xi–xiv** (egg fluid harvesting), 1–6 h depending on the number of samples
- **Substep xv** (check egg fluids for contamination), 1–3 days
- **Substep xvi** (clean up), 15 minutes
- **Substep xvii** (assess presence of IAV), see Part 3

Step 2, Option B: MDCK cell inoculation and supernatant harvest

- **Substeps i–iii** (MDCK preparation), 1 h plus a 16–24 h incubation
- **Substeps iv–ix** (MDCK inoculation), 2–3 h plus a 2–5 day incubation
- **Substep x** (monitor MDCK cells for CPE), 10–30 minutes
- **Substep xi** (harvest MDCK culture supernatants), 1–2 h
- **Substep xii** (MDCK passaging), see timing for substeps i–xi
- **Substep xiii** (cleanup), 15 minutes
- **Substep xiv** (assess presence of IAV), see Part 3

Part 3: Virus Detection by use of HA Assay, Standard RT-PCR, or Real-Time RT-PCR**Step 3, Option A: HA Assay**

- **Substeps i–v** (HA assay), 2 h

Step 3, Option B: Standard RT-PCR analysis

- **Substeps i–iii**, (RNA harvest), 30 minutes to 1 h
- **Substep iv** (one-tube RT-PCR reaction), 2.5–3 h
- **Substeps v–viii** (agarose gel electrophoresis), 2 h

Step 3, Option C: Real-time RT-PCR

- **Substep i**, (RNA harvest), 30 minutes to 1 h
- **Substep ii–v**, (cDNA synthesis and real-time PCR reaction), 2–3 h

Box 1: Further analysis of egg fluids

- **Substeps i–iv, part A**, (egg stock preparation), 1–2 h
- **Substep i–ii, part B**, (egg passaging), see timing for substeps i–xvii

TROUBLESHOOTING

See Table 1 for troubleshooting guidance.

ANTICIPATED RESULTS

Examples of typical assay results are provided in Figure 3 (cytopathic effects in MDCK cells), Figure 4 (HA assay) and Figure 5 (standard and real-time RT-PCR), and are described in more detail below.

Cytopathic effects in IAV-infected MDCK cells (Figure 3)

IAV-induced cytopathic effects in MDCK cells are characterized by cell rounding and detachment from the monolayer. For actively replicating cultures, this can be verified readily by the existence of floating cellular debris in the culture medium, a loss of monolayer coverage over the surface of the plate, and altered cellular morphology relative to mock-infected control monolayers (see Figure 3). In some cases, cytopathic effects may be more difficult to observe, particularly in earlier time points (e.g., see the CA04 panel of Figure 3), and so it is critical to always include a mock-infected control culture so that direct comparisons can be made. Complete loss of the monolayer is usually observed within 2–3 days post-inoculation (data not shown). It is important to note that bacterial or fungal contamination can result in the appearance of floating debris and, on occasion, changes in the cellular monolayer. Thus, personnel examining virus cultures should note drastic changes in pH and excessive turbidity of virus culture medium, as these observations strongly suggest the presence of undesirable contaminants.

HA assay (Figure 4)

The results of HA assays can be directly scored by eye and do not require the use of a plate reader. As described above, agglutination causes RBCs to remain in a sheet at the bottom of wells – giving a cloudy appearance – while the lack of agglutination results in RBC settling to the bottom of the well in the form of a button or halo surrounded by relatively translucent buffer (see Figure 4, **panel A** for examples of each of these phenomena). A sample that is authentically HA-positive will usually exhibit agglutination at multiple dilutions, and partial agglutination may be observed in HA-positive samples at dilutions that immediately precede the first negative dilution (Figure 4, **panel B**; see the wells highlighted by blue circles for examples of partial agglutination). Partially agglutinated positive samples should not be mistaken for negative halos, which are observed when using mammalian RBCs. To calculate a sample's HA titer, take the reciprocal of the highest dilution at which complete agglutination is observed. For example, if the highest completely agglutinated dilution is 2^{-7} , the virus is considered to have 128 HA units.

Standard RT-PCR (Figure 5, panel A)

The results of a standard RT-PCR assay are assessed by use of agarose gel electrophoresis of the PCR product. In Figure 5 (**panel A**), we show a typical result for the standard RT-PCR reaction using universal primers to the M gene, as described in this protocol. This PCR reaction detects M-gene fragments from IAVs of a variety of origins, although variability may be observed.

Real-time RT-PCR (Figure 5, panel B)

The results of real-time RT-PCR assays are determined by observing the normalized reporter (Rn) value for each reaction, which is calculated as the ratio of the fluorescence of the FAMTM dye divided by the fluorescence of a passive reference dye (ROXTM) at the end of each cycle. Results are reported as a Ct (cycle threshold) value, which is the number of cycles required for the Rn value to intersect the threshold line (established by negative control reactions), and is a relative measure of the concentration of target in the RT-PCR reaction. A typical Ct value for positive target amplification is 35.0, and the Rn value should exhibit a sigmoidal amplification curve. Any Ct signal above 35 cycles may be considered suspect and could require further confirmation. In Figure 5 (**panel B**), we show typical sigmoidal amplification curves for several different IAVs when using the real-time RT-PCR procedure described in this protocol. Some minor variability is noted between the Rn values of the viruses examined here (Figure 5, panel C). It should be emphasized that, while the primers for both the standard and real-time RT-PCR assays described in this protocol are ‘universal’ (i.e., designed to detect highly conserved M gene regions), it is possible that some IAVs may not be detected if primers are imperfectly matched to their M gene.

SUMMARY

The procedures described in this protocol allow the isolation, culture and identification of IAVs from different types of surveillance and research samples, and the resultant virus culture stocks can be retained and used in downstream analyses. For surveillance samples, additional subtype-specific RT-PCR (or other) assays may be performed to determine the specific HA and NA subtypes of isolated IAVs, and full genome sequencing may be employed to study the phylogenetic relationships of newly isolated viruses to known IAVs that originated in humans and/or reservoir species. Moreover, the assembled human surveillance data from multiple sources may be used by public health officials to determine the severity of a particular IAV season, to assess IAV vaccine efficacy, and to make recommendations for IAV vaccine strain selection for upcoming seasons. For H5N1-HPAI and H7N9 viruses isolated from animal surveillance samples, it is particularly important to identify genetic markers that are known to enhance virus growth and/or transmission in mammals (i.e., evidence for pandemic potential), and follow-up *in vivo* experiments should be performed to validate whether specific viruses exhibit pathogenicity or transmission in mammalian models of infection. In sum, this protocol is essential to enhance knowledge about IAVs that are currently circulating in humans and reservoir species, and to more clearly define potential IAV pandemic threats.

Acknowledgments

The authors are grateful to Masato Hatta, Shufang Fan, Jihui Ping, Zachary Najacht, and Alexander Karasin for helpful discussions, to Peter Jester and Zachary Najacht for assistance with HA assay and RT-PCR figure components, and to Susan Watson for scientific editing of the manuscript. This work was supported by grants-in-aid from the Ministry of Health, Labour, and Welfare, Japan, by ERATO (Japan Science and Technology Agency), by National Institute of Allergy and Infectious Diseases Public Health Service research grants, and by an NIAID-funded Center for Research on Influenza Pathogenesis (CRIP, HHSN266200700010C).

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Figure 1. IAV isolation, culture, and identification protocol flowchart

Protocol parts and subcomponents are indicated by the dark and light boxes, respectively. Specific text sections describing the steps for each technique are indicated at the right. The 'gold standard' workflow is shown by solid lines with arrows, and the 'fast' workflow is shown by dashed lines with arrows. Workflows start from the 'Part 1' box at the upper left.

Figure 2. Embryonated egg inoculation and allantoic fluid harvest

(A) A graphical longitudinal section of an embryonated egg's interior anatomy. "Em," embryo. (B) Photograph of a candled, 10-day-old embryonated chicken egg with an inoculation puncture. Anatomic regions, as described in (A), are indicated. (C) Photographs of (i) an 'in-house' egg piercing tool consisting of a 20-gauge, 1.5" catheter punched through a rubber stopper; (ii) the generation of the inoculation puncture by using the egg piercing tool; and (iii) the egg inoculation procedure. (D) Photographic representation of the egg allantoic fluid harvest procedure. After the egg was chilled at 4°C overnight, its shell

above the air sac was cracked and removed (*i*); the allantoic membrane was pulled back (*ii*); and the allantoic fluids were harvested (*iii*).

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Figure 3. IAV-induced cytopathic effects (CPE) in MDCK cells

MDCK cells were mock-infected (with MEM-BSA containing TPCK-trypsin but lacking any virus) or infected with 1:1000 dilutions of stock viruses of influenza A/Kawasaki/173/2001 (H1N1; ‘K173’), A/Yokohama/2017/2003 (H3N2; ‘Y2017’), or A/California/04/2009 (H1N1; ‘CA04’), as described in **Step 2, Option B, substeps i-x**. Twenty-four hours later, monolayers were examined for CPE by using a light microscope (4X objective; the red scale bar in the lower right of each panel represents a length of 200 μ M). Prominent and moderate CPE were observed in K173 and Y2017 infections, respectively. The blue arrow in the CA04 panel indicates minor CPE. By 48 h post-infection, all cells in all

infections were completely detached and floating in the cell culture medium (data not shown).

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Figure 4. Hemagglutination (HA) assay

(A) Samples lacking agglutinating activity (left panel) or containing IAV with agglutinating activity (center panel) were mixed with an equal volume of turkey RBCs (0.5%) in a U-bottomed microtiter plate and incubated for 30 minutes at room temperature; and a sample lacking agglutinating activity (right panel) was mixed with an equal volume of human RBCs (0.75%) in a U-bottomed microtiter plate and incubated for 1 hour at room temperature. At the end of each incubation period, 4X magnified images of individual microtiter plate wells were captured using a tissue culture microscope fitted with a digital camera. The left panel shows a characteristic negative 'button' result; the central panel shows the evenly distributed, 'cloudy' appearance of a positive agglutination result; and the right panel shows a thick ring of cells, i.e. a 'halo', negative result. (B) Samples containing IAV with agglutinating activity (see rows A, B, and D-G) or lacking IAV with agglutinating activity (see rows C and H) were subjected to an HA assay. Samples were 2-fold serially diluted (2⁰ – 2¹¹) in a 50-μl final volume, and then mixed with an equal volume of turkey RBCs (0.5%) in a U-bottomed microtiter plate. The entire microtiter plate was photographed after 30

minutes incubation at room temperature. The dilutions for each column and the corresponding HA units are indicated at the top of the panel, and the HA titer for each sample is indicated to the right. Wells exhibiting partial agglutination are indicated by dark blue circles.

Figure 5. RT-PCR Analysis

RNA was harvested from virus stocks of CA04 (H1N1), K173 (H1N1), Y2017 (H3N2), A/Vietnam/1203/2004 (H5N1; ‘VN1203’), and A/Anhui/1/2013 (H7N9; ‘AH1’), and the resultant RNA was subjected to standard RT-PCR (A) or real-time RT-PCR (B-C) procedures as described in this protocol. (A) Products from the standard RT-PCR reaction (1/3 of the total volume) were analyzed by agarose gel electrophoresis and an image of the gel is shown. The M-gene fragment location (~274 nt) is indicated by a bar at the right, sizes of double-stranded DNA standards are indicated at the left (Nt = nucleotides) and lane

descriptions – indicating the input template virus RNA – are provided to the right of the gel panel. (B) Real-time RT-PCR Rn/Ct plots for each virus template are shown. (C) The graph depicts the Ct value for each of the reactions plotted in panel (B). The dotted red line indicates the threshold for identification of a positive sample (the Ct value of the sample must be below this line to be confidently considered ‘positive’).

TABLE 1

Troubleshooting table

Step	Problem	Possible Reason	Solution
1 Part B Subpart vi	Tissue pieces do not break up well in the homogenizer.	(a) Tissue sections are too large. (b) The tube volume does not permit sufficient tissue mobility during the homogenization cycle.	(a) Reduce the tissue section size. (b) Reduce the media volume to 500 µl for the homogenization cycle, and add an additional 500 µl once the tissue has been completely dispersed.
2 Part A Subpart v	Difficulty inoculating allantoic or amniotic sacs	Inoculation must be done blindly.	Inexperienced researchers should practice egg inoculations with Coomassie stain (0.5%) prior to sample inoculations, and visually check where the stain settled to ensure appropriate inoculum targeting.
2 Part A Subpart xiii	Difficulty harvesting allantoic fluid	Albumin is trapped in the pipette.	Expel pipette contents and try to avoid albumin.
2 Part B Subpart iii	MDCK cells do not grow well in seeded cultures	Unhealthy MDCK cell cultures were used for seeding.	Avoid allowing cells to become over-confluent before passaging. Avoid using high passage number cells or expired cell culture medium. Check cells for mycoplasma contamination.
2 Part B Subpart xii	Lack of CPE in MDCK cultures	(a) The culture does not contain IAV. <i>If the inoculated sample is known to contain IAV:</i> (b) The IAV does not grow well in MDCK cells. (c) TPCK-trypsin was not included in the culture. (d) Inefficient monolayer washing prior to inoculation. (e) MDCK cells are not metabolically active.	(a) No further steps are required. (b) Passage the culture in MDCK cells to amplify any virus that is present, or repeat the inoculation using eggs. (c) Be sure to add TPCK-trypsin to the inoculation and virus culture medium. (d) Wash the monolayer three times before inoculation using PBS or MEM-BSA. (e) Avoid using cultures that are high passage number or overgrown. Avoid using expired cell culture medium. Only inoculate cultures at 80%–90% confluence.
3 Part A Subpart v	Difficulty discerning positive vs. negative HA assay results	(a) Microtiter plates were disturbed during the incubation period. (b) Flat-bottomed microtiter plates were improperly used.	(a) Avoid disturbing microtiter plates during the incubation period. (b) Only use U-bottomed plates for HA assays.
3 Part B Subpart viii and 3 Part C Subpart v	Lack of M gene amplification	(a) The culture does not contain IAV. <i>If the inoculated sample is known to contain IAV:</i> (b) RNA isolation was not successful. (c) Primer mis-match occurred between the conserved M gene sequence of the primer and that of M gene in the sample. (d) PCR reagents are degraded or expired.	(a) No further steps are required. (b) Assay RNA levels of RNeasy preps by using spectrophotometry. Check for amplification of M gene sequences in a positive control prepared in parallel. (c) Check for amplification of M gene sequences in a positive control prepared in parallel; use an alternative set of primers (d) Repeat the assay using new reagents.
Box 1 Part B Subpart ii	Lack of agglutinating activity in egg fluids	(a) The culture does not contain IAV. <i>If the inoculated sample is known to contain IAV:</i> (b) The IAV does not grow well in eggs. (c) The egg was inoculated improperly. (d) The IAV in question does not agglutinate RBCs derived from the species in use.	(a) No further steps are required. (b) Passage the culture in eggs to amplify any virus that is present, or repeat the inoculation using MDCK cell cultures. (c) See the solution for Step 2, Part A Subpart v. (d) Repeat the HA assay with RBCs from another species.