

Laboratory Testing

Laboratory testing requires staff with expertise in the methods performed, adequate space with appropriate biosafety controls, well-maintained equipment, appropriate testing reagents and supplies, and reliable results reporting mechanisms. Essential equipment, materials, and reagents necessary to perform influenza virologic surveillance are listed in Appendix C: Laboratory Methods.

Influenza virologic testing methods are classified in the roadmap based on a stakeholder assessment of requirements that should be maintained and available at all PHLs involved in influenza surveillance (primary testing); or as additional influenza surveillance laboratory capabilities that may be maintained based on state and/or jurisdictional needs or provided through a shared services model. The laboratory must ensure quality for all influenza testing methodologies performed.

Primary Testing Method

PHLs performing virologic surveillance are expected to utilize molecular methods, such as real-time reverse transcriptase PCR (rRT-PCR), as the primary testing method for influenza detection and subtyping. This is an ELC benchmark. During rRT-PCR test processes, viral RNA is extracted from patient specimens, transcribed into DNA and then amplified. The product of the test reaction is detected in “real time” using labeled probes. Real-time RT-PCR can rapidly identify influenza A and B, distinguish influenza A subtypes, and offers the best performance characteristics (i.e., sensitivity, specificity) of all currently available testing methods. CDC, through the Influenza Reagent Resource (IRR), manufactures and distributes the CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel (CDC Flu rRT-PCR Dx Panel) to all state and qualified local PHLs engaged in influenza surveillance testing to ensure widespread use of a nationally standardized protocol. The CDC also has the capability to rapidly adapt the assay to detect newly emerging viruses. The CDC Flu rRT-PCR Dx Panel is distributed under FDA special controls. The special controls require that all users of the diagnostic device be trained to perform and interpret the results by a competent instructor prior to use and that CDC limit distribution to only those users who have successfully completed a training course provided or approved by CDC.

If the PHL decides not to use the CDC Flu rRT-PCR Dx Panel for influenza surveillance testing but instead chooses an alternative molecular influenza testing methodology or platform, the PHL must ensure that the manufacturer of the assay monitors and updates assay performance as circulating viruses change over time and that the assay being utilized within the laboratory has been optimized to identify all currently circulating influenza viruses.

Influenza testing algorithms should be adopted in order to optimize testing efficiency and throughput. Laboratories should choose an algorithm that best fits their laboratory test flow, and surveillance needs as the influenza prevalence changes throughout the year, and ensures judicious use of CDC provided reagents. Algorithms should be continually reviewed by PHLs throughout the influenza season to ensure that the most efficient algorithm is being utilized. The direct material cost to CDC for each IVD CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel, including Influenza A/B typing and subtyping reagents, enzymes, extraction kits and plastics, is approximately \$14,000 (i.e., \$20 for each specimen tested by the PHLs).

Below are three common testing algorithms to consider.

- Using the 7-target algorithm, influenza typing and subtyping are performed on a single plate at the same time. This algorithm tests simultaneously for influenza A, B, H1, H3, A/H1pdm2009 and RNaseP (internal control).
- The Influenza A/B Reflex algorithm is a two-step process in which Influenza A/B typing (A, B, RNaseP) is performed first and the Influenza A subtyping (H1, H3, A/H1pdm2009) is performed only on Influenza A positive specimens.
- Surge algorithms can also be implemented if needed to accommodate high volume testing demand in an outbreak investigation or pandemic. This may include first screening all specimens associated with outbreak for the outbreak strain (e.g., A, H1pdm2009), followed by reflex testing of negative samples to detect other circulating influenza viruses.

The PHL is responsible for the timely referral of representative specimens (and viruses, if culture is performed) to CDC or a CDC-designated PHL for genetic and antigenic characterization throughout the year. PHLs should submit 1mL of original clinical material (a minimum volume of 300µl is required). To enhance CDC's vaccine virus selection efforts, it is important to routinely and consistently send recently collected specimens. Specimens submitted to CDC should be representative of the circulating influenza types/subtypes, geography, disease severity and age. Oversampling of low prevalence subtypes may be necessary to ensure that all circulating subtypes are represented in the samples sent to CDC. When available, viruses from particularly severe or unusual cases, and a sample of viruses isolated from outbreak investigations should also be represented in submissions to CDC. The two examples below illustrate the criteria that should be considered by the PHL when selecting the specimens that will be sent to CDC for routine national surveillance purposes.

At any time through the year, if the PHL identifies a specimen as unsubtypeable following testing using all available targets (influenza A, B, H1, H3, H1pdm2009, etc), the laboratory must notify CDC immediately and refer the specimen to CDC within 24 hours of detection. Specific information about interpretation of inconclusive test results and referral requirements can be found in the CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel package insert.

- In a two week period, the PHL testing yields 50 A/H3, 1 A/H1pdm2009 and 5 influenza B positive specimens. Send to CDC or the CDC-designated laboratory: the A/H1 specimen, 1 influenza B, and 3 A/H3 viruses that are representative of state geography and patient ages.
- In a two week period, the PHL testing yields 20 A/H3, one of which is from a patient who died, 12 A/H1pdm2009 viruses and 5 Influenza B viruses. Send to CDC or the CDC-designated laboratory: the A/H3 specimen from the patient who died, 2 other representative A/H3 specimens, 2 A/H1pdm2009 and 1 influenza B specimen.

Outside of influenza season, PHLs should follow CDC summer submission guidance and send all specimens that test positive by PCR to CDC for further characterization.

IMPORTANT REPORTING AND REFERRAL REQUIREMENT: At any time through the year, if the PHL identifies a specimen as unsubtypable following testing using all available targets (influenza A, B, H1, H3, H1pdm2009, etc), the laboratory must notify CDC immediately and refer the specimen to CDC within 24 hours of detection. Specific information about interpretation of inconclusive test results and referral requirements can be found in the CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel package insert.

To ensure national and state coordination on virologic surveillance priorities, “Influenza Seasonal Kick off Teleconferences” are convened for PHLs, influenza coordinators, and state epidemiologists in the early fall each year. The main purpose of the teleconferences is to provide a situational update and surveillance guidance for the upcoming season, including expectations for submission of specimens to CDC. The guidance will identify how many specimens to submit throughout the season, where to submit the specimens, and the specimen submission form.

Additional Testing Methods

Additional testing methods include influenza virus culture, antiviral resistance testing, influenza hemagglutination inhibition (HAI), immunofluorescence testing, and serology. The methodology recommendations presented here focus specifically on advantages and disadvantages for influenza and may not apply to other virus assays. Each testing methodology listed has many advantages and disadvantages that will factor into jurisdictional test methodology decisions. Some of the advantages or disadvantages listed here are more applicable to national surveillance needs, but are included to help inform jurisdictional decisions.

- **Influenza Virus Culture**

PHLs are encouraged to perform virus culture if they can sustain the appropriate level of staff expertise and the necessary resources. When PHLs submit virus isolates along with clinical material to CDC or the CDC-designated PHL, this improves the efficiency of the antigenic characterization process at CDC, so that information regarding influenza vaccine match or virus drift can be disseminated back to PHLs more quickly.

At minimum this capability must be maintained at CDC, and at a subset of state PHLs. PHLs that do not have the capability or resources to perform high quality virus culture using consensus protocols may opt to utilize a shared services model instead, relying on the CDC or state PHLs that have been designated by CDC as national surveillance laboratories to provide virus culture.

Advantages of Maintaining or Implementing Influenza Virus Culture:

PHL:

- Provides isolates for validation and verification of new or modified assays, and troubleshooting investigations.
- Provides a back-up method to PCR.
- Detects other respiratory viruses if additional cell lines are used.
- Provides viruses required for phenotypic antiviral resistance testing.

CDC:

- Provides isolates for validation and verification of new or modified assays, and troubleshooting investigations.
- Provides viruses required for phenotypic and antigenic characterization. These are critical components of surveillance for vaccine virus strain selection, and development of the annual WHO kit distributed to domestic and international laboratories.
- Provides viruses that can be used to develop vaccine candidates.

Disadvantages of Influenza Virus Culture:

- Less rapid and sensitive than rRT-PCR and is not efficient in times of surge.
- Requires specialized expertise and capability to maintain high quality cell lines.
- Variable growth characteristics and sensitivity with influenza strains in different cell lines.

Minimal Implementation Considerations:

- Utilize standard reference methods such as those described in the “WHO Manual for the Laboratory Diagnosis and Virological Surveillance of Influenza”.²⁷
- Ensure sustainable expertise. Each laboratory must have a training plan in place for virus isolation methods and troubleshooting.
- Establish a backup plan for times when commercially available cells are not available.
- Perform an accurate cost analysis. Identify sustainable sources of funding.

- **Antiviral Resistance Testing (Pyrosequencing, Neuraminidase Inhibition)**

Definitive antiviral resistance testing requires both phenotypic resistance testing of the virus using a neuraminidase inhibition (NAI) assay, and detection of genetic markers of drug resistance by pyrosequencing and/or sequencing. Both of these test methods are performed at CDC. Pyrosequencing is also performed at a subset of PHLs; this provides a cost-effective and efficient approach to expand antiviral resistance surveillance, providing data that can be used to inform patient management and treatment recommendations at the regional and national level.

Advantages of Maintaining or Implementing Pyrosequencing:

PHL and CDC:

- Detects established and potential genetic markers of resistance.
- Provides more rapid turn-around-time than NAI which requires propagated virus for testing.

Disadvantages of Pyrosequencing:

- Requires multiple assays to detect different genetic markers for each influenza subtype (e.g., H275Y is the marker for only A/H1pdm 2009).

- Requires supplemental testing by NAI for definitive confirmation of antiviral resistance.
- Requires specialized expertise to perform and interpret results.
- Requires specialized equipment and expensive reagents.
- Requires periodic revalidation of assays due to frequent genetic changes.
- Requires knowledge of molecular markers of resistance which may not be available for a novel virus and new NAIs.

Advantages of Maintaining or Implementing NAI:

PHL and CDC:

- Detects resistance that is not caused by previously identified genetic markers of resistance.
- Detects resistance in novel viruses for which genetic markers of resistance are unknown.
- Detects resistance to a new NAI drug for which genetic markers of resistance are unknown.

Disadvantages of NAI:

- Requires specialized expertise to perform and interpret results.
- Requires specialized equipment and expensive reagents.
- Requires special formulations of antiviral drugs (e.g., oseltamivir carboxylate) not available outside of drug manufacturer.

Minimal Implementation Considerations:

- Perform an accurate cost analysis. Identify sustainable sources of funding.
- Laboratory must have the capability to:
 - Interpret and report test results appropriately for either virologic surveillance, or patient management.
 - Report all results to CDC in a timely manner for inclusion in national antiviral resistance surveillance data.
 - Revalidate assays when mutations occur or changes to the protocols are made.
 - Perform virus isolation if using NAI assay.
 - Submit all influenza viruses positive for any resistance marker to CDC in a timely manner for confirmatory testing.

- **Respiratory Pathogen Assays**

Molecular testing for other respiratory viruses, such as commercially available RVP assays or laboratory developed tests (LDTs), have become common in many clinical laboratories and are increasingly used in PHLs to provide jurisdictional and national information about other circulating respiratory pathogens. These data can aid in identifying other agents that cause influenza like community illnesses or outbreaks and provide reassurance that surveillance is not “missing” influenza during periods when influenza activity is low but other influenza like illnesses are prevalent.

Advantages of Maintaining or Implementing Molecular Respiratory Pathogen Assays:

PHL:

- Provides data on circulating respiratory viruses that can be used for state, regional or national (NREVSS) surveillance or outbreak investigations.
- Provides a more rapid, sensitive and specific alternative to respiratory virus culture.
- Provides influenza surveillance partners with additional information about circulating viruses that may cause ILI, this may also help to incentivize partners to consistently contribute to flu surveillance.

Disadvantages of Respiratory Pathogen Assays:

- Cost.
- Variable sensitivity and specificity across pathogens and commercial assays.
- May lose sensitivity overtime, especially for influenza, due to changes in the viruses.

Minimal Implementation Considerations:

- Perform an accurate cost analysis. Identify sustainable sources of funding.
- Determine how respiratory pathogen data will be used to supplement influenza surveillance data and/or diagnostic testing.

- **Influenza Hemagglutination Inhibition Test**

Influenza hemagglutination inhibition (HI or HAI) test performed at CDC, using strain specific ferret antisera, remains a test of choice for antigenic characterization to monitor changes in circulating influenza viruses and inform influenza vaccine virus selection. HAI testing using the WHO kit reagents that are provided by CDC to the PHLs can be used to detect and identify influenza viruses, but not for monitoring antigenic drift of influenza viruses.

Advantages for Maintaining or Implementing Influenza HAI test:

PHL:

- Identifies influenza type, influenza A subtype, and influenza B lineage using the Influenza WHO kit reagents provided by CDC.
- Facilitates and helps to ensure more efficient detection of antigenic variants.

CDC:

- Provides information on antigenic changes that may impact vaccine effectiveness, these data are critical to inform annual vaccine virus selection.

Disadvantages of Influenza HAI test:

- Requires significant expertise and specialized reagents (e.g., turkey red blood cells).
- Requires virus culture.
- Time consuming.
- Influenza type and subtype can more rapidly be determined in PHLs using rRT-PCR.

Minimal Implementation Considerations:

- Laboratory must have the capability to:
 - Interpret and report test results appropriately for virologic surveillance.
 - Perform virus isolation.
 - Obtain red blood cells from appropriate species (e.g., turkey).
- Perform an accurate cost analysis. Identify sustainable sources of funding.

- **Influenza Serologic testing**

Serologic testing is neither a surveillance nor rapid diagnostic testing tool but is currently used primarily by CDC and academic institutions for vaccine effectiveness studies, annual vaccine strain selection, research purposes or retrospective seroprevalence studies. Serologic testing to detect influenza virus antibodies may be performed using a variety of methods, including hemagglutination inhibition, enzyme-linked immunosorbent assay and microneutralization.

Advantages of Maintaining or Implementing Influenza Serologic Testing:**PHL and CDC:**

- Provides serology data to inform virologic strain selection.
- Provides serological diagnosis and retrospective seroprevalence data.

Disadvantages of Influenza Serologic Testing:

- Requires capability to implement assay as a laboratory-developed test, there are no FDA cleared methods for influenza serology.
- Requires both a high level of expertise and specialized reagents.
- Interpreting results is difficult and patient serologic responses may be cross-reactive.

Minimal Implementation Considerations:

- Perform an accurate cost analysis. Identify sustainable sources of funding.
- Maintain staff with capability to interpret serologic test results in the context of surveillance.

- **Direct Specimen Immunofluorescence**

Direct specimen immunofluorescence using direct fluorescent antibody (DFA) testing methods involve testing clinical material taken directly from the patient. These cell preparations are tested using fluorescent-labeled antibodies, which are visible when examined using an immunofluorescence microscope. DFA is primarily a diagnostic tool.

Advantages of Maintaining and Implementing DFA:

PHL:

- Provides the ability to produce results within an hour of specimen receipt.

Disadvantages of DFA:

- Requires specialized expertise and fluorescent microscopes.
- Results are subjective and dependent on the individual reader's expertise.
- Not as accurate as rRT-PCR.
- Sensitivity and specificity of reagents may vary in response to current strains.

Minimal Implementation Considerations:

- Assess the necessity of maintaining this as a diagnostic tool.
- Perform an accurate cost analysis, identify sustainable sources of funding.

- **RIDTs**

Currently available RIDTs for the detection of influenza viruses employ a variety of methods, including enzyme-linked immunosorbent assays, immunochromatographic lateral flow immunoassays, and membrane-based immunoassays. In addition to differences in methodologies, these tests also have varying requirements for specimen collection and handling.

Advantages of Maintaining and Implementing RIDTs:

PHL:

- Produce rapid results for patient care, often within fifteen to thirty minutes.
- Have widespread commercial availability.
- Require minimal training, can be performed in non-laboratory settings.

Disadvantages of RIDTs:

- Significantly less sensitive and specific than rRT-PCR assays.
- Potentially less reliable when new virus strains emerge.

Minimal Implementation Considerations:

- Determine how RIDT data will be used to supplement influenza surveillance data and/or diagnostic testing.
- Establish policies for reflex/confirmatory testing.
- Perform an accurate cost analysis. Identify sustainable sources of funding.

Considerations for Maintaining or Implementing Influenza Testing Methodologies

In addition to considering the advantages and disadvantages of the various test methods, below is a set of questions that can be used as a decision tool in deliberations among the laboratory director, senior infectious disease laboratory staff, epidemiologists, influenza surveillance coordinator, and clinical laboratory partners.

1. Which influenza tests are most important to maintain?
2. For what purpose is the influenza test(s) needed?
3. How will the test results contribute to influenza virologic surveillance data within the state?
4. How is the influenza test methodology in question currently funded? Is this funding mechanism sustainable?
5. Is there an expectation, either through official policy or relations with the local clinical community, that the laboratory maintain capacity to perform some or all of the “additional” influenza tests?

Performance Accuracy of RIDT’s

RIDTs may be used to help with diagnostic and treatment decisions for patients in clinical settings, such as whether to prescribe antiviral medications. However, due to the limited sensitivities and predictive values of RIDTs, negative results of RIDTs do not exclude influenza virus infection and influenza should still be considered in a patient if clinical suspicion is high based upon history, signs, symptoms and clinical examination.³¹

Reported sensitivities of RIDTs range from 10-80% compared to viral culture or rRT-PCR. Specificities of RIDTs are approximately 90-95% (range 85-100%). RIDT users should be especially aware of the potential limitations of these tests to detect novel influenza viruses.^{8,32}

rRT-PCR to confirm results of an RIDT are recommended when:

- patient tests negative by RIDT when community influenza activity is high and laboratory confirmation of influenza is desired.
- patient tests positive by RIDT, the community prevalence of influenza is low and a false positive result is a consideration.
- patient has had recent close exposure to pigs or poultry or other animals and novel influenza A virus infection is possible.

6. If yes, what are the reasons for this expectation? For example, is there a perceived need to maintain “traditional methods” in the PHL as the capability to perform culture and HAI declines in the clinical laboratory sector?
7. Is the necessary expertise available in the PHL and is it sustainable? Does the laboratory have an experienced microbiologist/virologist that understands the basic virology, epidemiology, pathogenesis of influenza and other respiratory pathogens to enable test result and surveillance data interpretation, and surveillance capability decisions?
8. Does the laboratory have access to and utilize appropriate consensus protocols and testing materials (e.g., MDCK cell lines, antiviral drugs, monoclonal fluorescent antibodies)?
9. If an influenza test method, such as culture, is eliminated, do decision makers understand that implementing testing on an as needed basis would be extremely difficult due to loss of expertise, length of time needed to acquire testing supplies, and time to perform CLIA required validation studies?
10. Is the influenza methodology that is deemed necessary available from another source, such as CDC, a PHL shared services site, or a hospital/academic laboratory? What are the concerns with and road blocks to accessing alternate sources of testing?
11. Do the resources (funding, staffing, supply costs) needed to maintain the additional test methods adversely impact the capacity to perform rRT-PCR and test the number of specimens necessary for routine surveillance, or other essential laboratory functions?
12. If the IRR was unable to continue providing either some (i.e., ancillary) or all the reagents needed for rRT-PCR, how would this affect the decision to maintain “additional” testing methods?
13. Does the laboratory meet the minimal considerations listed for each test method that will be implemented or maintained?