Sample-to-result Nucleic Acid Test Enables Accurate Detection of Influenza A/2009 H1N1 in 26 Minutes in Near-patient Settings

Yu Tian, Kiran Madanahally, Hema Rao, Reena Mackwan and Lingjun Chen

IQuum, Inc.

Abstract

The Liat™ Influenza A/2009 H1N1 Assay (IQuum, Inc., Marlborough, MA) is an automated molecular diagnostic test for the qualitative detection and differentiation of Influenza A and 2009 H1N1 viral ribonucleic acid (RNA) from nasopharyngeal swab samples in 26 minutes. Performed on the Liat™ Analyzer, the assay received the US Food and Drug Administration (FDA) Emergency Use Authorization (EUA, expired June 23, 2010) for 2009 H1N1 detection in laboratories certified under the Clinical Laboratory Improvement Amendments (CLIA) of 1988 to perform moderate- or high-complexity tests. In analytical studies, the assay demonstrated a limit of detection of 10^{-2}–10^{-1} $TCID_{50}$/ml of influenza virus depending on the strain. The assay further showed appropriate reactivity against all 23 influenza A strains tested, including seasonal influenzas and 2009 H1N1 viruses. No cross-reactivity with 16 bacteria, 15 non-influenza viruses and 10 influenza B viruses was also demonstrated. Clinical sample testing showed 100% positive and 100% negative agreement with comparator EUA assays or viral culture in 226 clinical samples tested (lower 95% confidence internal [CI] >94.4%). While maintaining substantially equivalent performance as high-complexity laboratory-based nucleic acid tests, the Liat Influenza A/2009 H1N1 Assay demonstrated the capability to bring nucleic acid testing to substantially the same speed and simplicity as lateral flow immunoassays.

Keywords

Influenza, H1N1, point-of-care, near-patient, molecular diagnostics, rapid nucleic acid test

Disclosure: The authors are employees of IQuum, Inc.

Acknowledgement: This work was partially funded by the National Institute of Allergy and Infectious Diseases (NIAID), Small Business Innovation Research (SBIR) Grant Number 5U01AI082187-02.

Received: 2 September 2010 **Accepted:** 28 September 2010 **Citation:** *European Infectious Disease*, 2010;4(2):26–30

Correspondence: Lingjun Chen, IQuum, Inc., 700 Nickerson Road, Marlborough, MA 01752, US. E: lingjun@iquum.com

Support: The printing costs for the publication of this article were funded by IQuum, Inc.

In Vitro Diagnostic Assays for 2009 H1N1 Influenza

In April 2009 the US Centers for Disease Control and Prevention (CDC) identified a novel influenza A virus in samples from two cases in the US and retrospective cases in Mexico.[1–3] The virus contains genetic elements from two different types of swine influenzas, as well as elements originally from avian and human influenzas that were incorporated into other swine influenza viruses.[4] While swine flu viruses with avian, human and swine genes have previously infected humans, those infections have not transmitted efficiently from human to human.[5,6,7] In contrast, this novel virus not only infected humans and caused disease, but also had sustained ability for efficient human-to-human transmission, even into the summer months. This novel 'swine flu' 2009 H1N1 strain rapidly spread to 214 countries and killed 18,337 people worldwide as of 12 July 2010.[8] As a result of the ease of infection, lack of immunity in the human population and unavailability of diagnostic and response tools, the World Health Organization (WHO) declared a global pandemic in July 2009.[9]

To identify and respond to the pandemic outbreak, the US Food and Drug Administration (FDA) announced the Emergency Use Authorization (EUA) mechanism to rapidly make available diagnostic and therapeutic tools for 2009 H1N1. While both lateral flow immunoassays and nucleic acid tests (NAT) existed for influenza, such tests were not able to specifically detect 2009 H1N1 and distinguish it from other seasonal influenza A viruses. Influenza detection has usually been performed using immunoassays in physician's offices and hospitals or nucleic acid testing or viral culture in centralised laboratories. While the simplicity and rapidness of influenza immunoassays make them convenient for use in near-patient settings, their relatively poor clinical sensitivity has typically caused a high rate of false-negative results, leading to the requirement to confirm negative results by viral culture.[10,11]

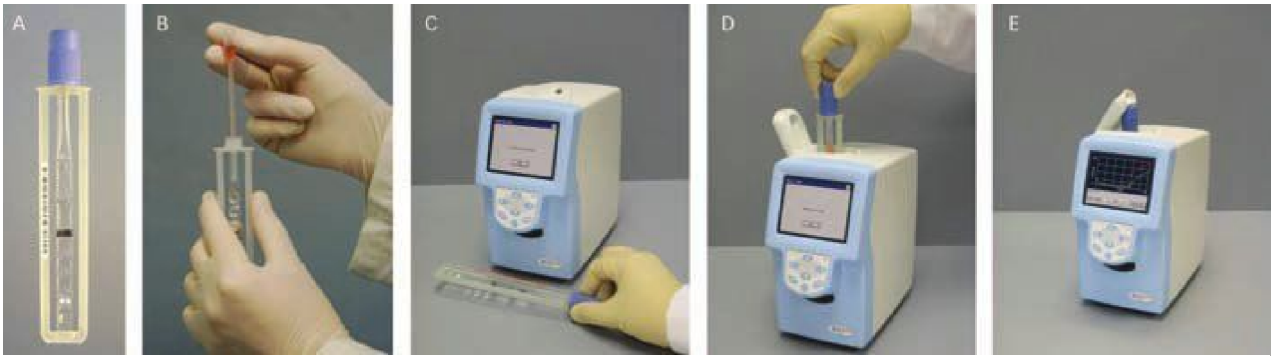
On the other hand, nucleic acid tests have demonstrated superior sensitivity and specificity. However, their technical complexity and susceptibility to contamination require such assays to be performed in certified clinical laboratories by well-trained specialists.[12] In the US, Clinical Laboratory Improvement Amendments (CLIA) authorises the FDA to certify tests according to the technical complexity and risk of reporting erroneous results. Nucleic acid tests have typically been categorised as high-complexity. As a result, such tests can only be performed in the relatively few number of high-complexity-certified laboratories, which must meet stringent requirements for proficiency testing, patient test management, quality control, quality assurance

Table 1: Comparison of Liat Influenza A/2009 H1N1 Assay to Other Assays Receiving US Food and Drug Administration Emergency Use Authorization

Company	IQuum	Focus Diagnostics	US Centers for Disease Control & Prevention	Roche Diagnostics
Item Name	Liat Influenza A/2009 H1N1	Influenza A H1N1(2009) RT-PCR	CDC Swine Influenza Virus RT-PCR Panel	Realtime ready Influenza A/H1N1 Detection Set
Assay target	Influenza A 2009 H1N1 Influenza	Influenza A 2009 H1N1 influenza	Influenza A, swine influenza A swine H1N1	Influenza A 2009 H1N1 influenza
Assay platform	Liat Analyzer	Applied Biosystems 7500 RT-PCR system	Applied Biosystems 7500 Fast Dx RT-PCR or Roche Light Cycler® 2.0 RT-PCR systems	Light Cycler® 2.0 instrument
Self-contained system	Yes	No	No	No
Fully automated	Yes: sample prep, amplification, detection and result interpretation	No: multiple manual steps required	No: multiple manual steps required	No: multiple manual steps required
Extraction method	Integrated silica-magnetic bead based nucleic acid extraction	Roche MagNA Pure LC Instrument and MagNA Pure LC Total Nucleic Acid Isolation Kit	Roche MagNA Pure LC Instrument and MagNA Pure LC Total Nucleic Acid Isolation Kit or Qiagen QIAamp® Viral RNA mini or RNeasy® mini kit	Roche MagNA Pure LC Instrument and MagNA Pure LC Total Nucleic Acid Isolation Kit
Assay method	PCR-based system for detecting the presence/absence of viral RNA in clinical specimens	Same	Same	Same
Result interpretation	Automated	Manual	Manual	Manual
User	Hospital nurse and moderate-complexity laboratory technologist	High-complexity laboratory technologist	High-complexity laboratory technologist	High-complexity laboratory technologist
Time-to-result	~26 minutes	~3 hours	~3 hours	~3 hours

CDC = Centers for Disease Control and Prevention; RNA = ribonucleic acid; RT-PCR = realtime polymerase chain reaction.

Figure 1: Liat Analyzer Operation



A sample such as a nasopharyngeal swab specimen is input directly into a Liat tube (A, B). After the tube is capped, the analyser scans the tube barcode (C) and the tube is inserted into the Liat Analyzer (D). The Liat Analyzer then automatically performs all nucleic acid test steps, including sample preparation, nucleic acid extraction, amplification and realtime detection, and reports results in 26 minutes (E).

and personnel. Additionally, the assays take at least a few hours to perform because of the labour-intensive sample preparation and nucleic acid purification processes, as well as the time-consuming nucleic acid amplification reactions. Such issues mean that these assays are not ideal for pandemic response, where immediate and on-site detection is key to controlling the spread of infection.

To overcome these respective challenges, the Liat™ Influenza A/2009 H1N1 Assay was developed to be a highly sensitive and specific nucleic acid test for influenza viral ribonucleic acid (RNA) detection, while maintaining the same level of simplicity and speed as immunoassays. The Liat Influenza A/2009 H1N1 Assay received US FDA EUA as a moderate-complexity test,[13,14] enabling use at a larger number of decentralised sites. With a turnaround time of only 26 minutes, the assay is the fastest nucleic acid test authorised by the US FDA. At the same time, the assay demonstrated substantially equivalent performance compared with other high-complexity nucleic acid tests.

Liat™ Influenza A/2009 H1N1 Assay Description

The Liat Influenza A/2009 H1N1 Assay is a rapid, automated, multiplex realtime polymerase chain reaction (RT-PCR) assay for the *in vitro* qualitative detection and differentiation of 2009 H1N1 influenza viral RNA. The Liat Influenza A/2009 H1N1 Assay uses nasopharyngeal swab (NPS) specimens collected from patients with signs and symptoms of respiratory infection in conjunction with clinical and epidemiological risk factors.

Table 2: Clinical Samples Tested by Sample Type and Comparator Test Method

Clinical Sample	Focus Diagnostics[a]	CDC[b]	Roche Diagnostics[c]	Viral Culture	Validate RT-PCR Assay[d]	Total Samples
Inf A/2009 H1N1	35	30	N/A	N/A	N/A	65
Inf A non-2009 H1N1	N/A	N/A	26	15	20	61
Inf A Negative	50	5	N/A	45	N/A	100

All 226 clinical samples were tested on the Liat Influenza A/2009 H1N1 Assay to evaluate the clinical performance of the assay.
a: Focus Diagnostic Influenza A H1N1(2009) Realtime Polymerase Chain Reaction (RT-PCR) Assay; b: CDC Swine Influenza Virus Realtime RT-PCR Panel; c: Roche Diagnostics Realtime Ready Influenza A/H1N1 Detection Assay; d: RT-PCR assay developed and validated by New York State Department of Health (Albany, NY).
CDC = Centers for Disease Control and Prevention; Inf = influenza; N/A = not applicable RT-PCR = realtime polymerase chain reaction.

The Liat Influenza A/2009 H1N1 Assay is performed on the Liat Analyzer, a small (~4.3x8-inch footprint) standalone instrument that automates and integrates all NAT processes, including reagent preparation, target enrichment, inhibitor removal, nucleic acid extraction and RT-PCR amplification and detection of target sequences. The assay detects a conserved region of the matrix gene of influenza A viral RNA (Inf A target) and the haemagglutinin gene of 2009 H1N1 viral RNA (2009 H1 target). An internal process control (IPC) is also included in the assay to monitor the processing of the target viruses and prevent false negative results due to the presence of inhibitors in the RT-PCR reaction. Each Liat tube is self-contained; therefore, cross-contamination between samples is minimised. Turnaround time for the test is 26 minutes. *Table 1* shows a comparison between the performance features of the Liat assay and other EUA assays used as comparator tests in this study.[14–17]

Liat System Operation

The Liat system refines the testing process to three simple steps: collecting a raw biological sample such as a nasopharyngeal swab specimen into a Liat assay tube and capping the tube; scanning the tube's barcode to identify the test and track the patient sample; and inserting the tube into the Liat Analyzer (see *Figure 1*). The analyser automatically executes all required assay steps and reports interpreted test results on the built-in touch screen in 26 minutes. No reagent preparation, manual processing or other operator intervention or result interpretation is required.

In the Liat Analyzer, multiple sample processors are aligned perpendicular to the Liat tube. Each sample processor comprises a temperature control element to heat, cool or incubate the sample within the Liat tube, and an actuator to compress the Liat tube to manipulate the sample, sequentially move the sample from one segment to another, and burst the peelable seals to selectively release reagents from the tube segments. An embedded microprocessor controls and co-ordinates the action of these sample processors to enable the system to perform all required assay processes within the Liat tube. For example, magnetic beads can be incubated with a sample for nucleic acid target enrichment and then captured and washed to remove possible inhibitors. Subsequently, nucleic acids can be eluted from the beads and transferred alternately between tube segments at different temperatures for rapid PCR amplification and real-time detection. The internal optical system provides six independent optical detection channels for multiplex detection and inclusion of internal controls in each test. Embedded sensors and error diagnostic software continuously monitor analyser function to detect and correct internal errors. The Liat Analyzer's integrated colour liquid crystal display (LCD) touch-screen further provides an easy-to-use interface for input and result display. In addition, the analyser's network capability enables report printing and connectivity to laboratory information systems, as well as remote system diagnostics and performance monitoring.

Analytic Performance

To determine the performance characteristics of the Liat Influenza A/2009 H1N1 Assay, the assay was first tested using spiked samples in analytical studies. Specifically, the limit-of-detection, reactivity and cross-reactivity of the assay were determined.

Limit-of-detection

The limit-of-detection (LOD), or analytical sensitivity, is defined as the lowest concentration of virus at which 95% of the tested replicates are detected as positive.[18] The LOD of the Liat assay was determined by limiting dilution studies using titered cultures of 2009 H1N1 viruses (A/Swine NY/01/2009 H1N1, A/Swine NY/02/2009 H1N1) and seasonal influenza A viruses (A/Brisbane/59/07) spiked in negative sample matrix. LOD is reported at the lowest concentration at which at least 19 out of 20 runs (>95%) reported positive detection. The test results showed the LOD for A/Swine NY/01/2009 H1N1, A/Swine NY/02/2009 H1N1 and A/Brisbane/59/07 strains were 10^{-2} $TCID_{50}$/ml, 10^{-1} $TCID_{50}$/ml and 10^{-1} $TCID_{50}$/ml, respectively. For reference, the CDC Swine Influenza Virus RT-PCR Detection Panel demonstrated an LOD of $10^{1.6}$ to 10^{3} $TCID_{50}$/ml, while the Realtime Ready Influenza A/H1N1 Detection Set showed an LOD of 10^{1} to 10^{3} $TCID_{50}$/ml, although different influenza strains were used for LOD determination.[16,17] Overall, the Liat assay demonstrated equivalent or better analytical sensitivity compared with other EUA RT-PCR assays.

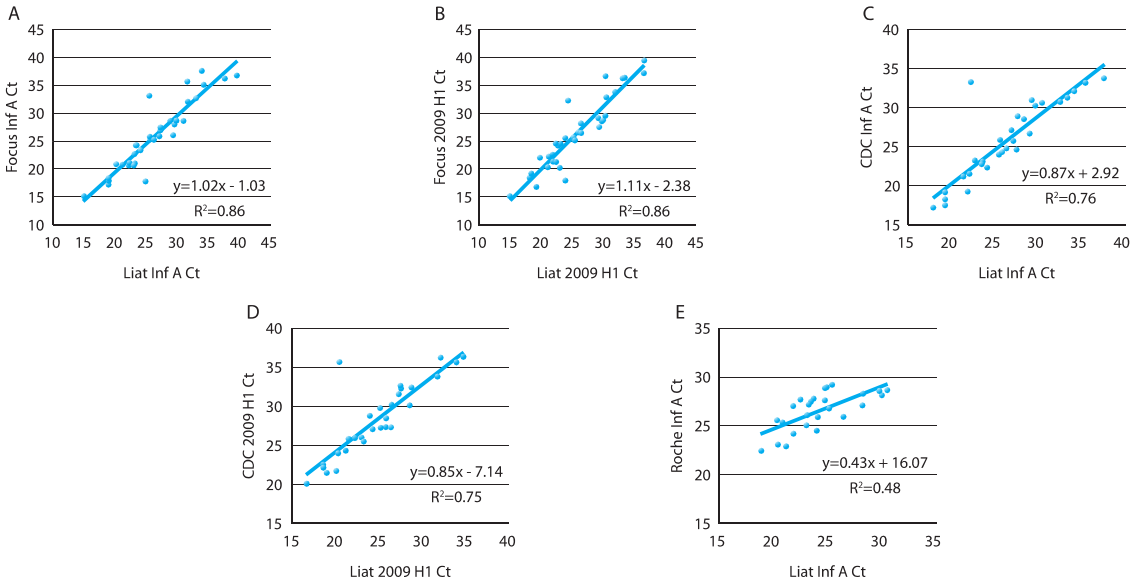
Analytical Reactivity

Reactivity studies evaluate the ability of the assay to specifically detect representative influenza strains.[19] Analytical reactivity was tested using 23 Influenza A strains at approximately 10^{2} $TCID_{50}$/ml. Strains tested included: 2009 H1N1 strains (A/Swine NY/01/2009 H1N1, A/Swine NY/02/2009 H1N1, A/Swine NY/03/2009 H1N1); non-2009 A/H1 strains (A/Brisbane/59/07, A/PR/8/34, A/FM/1/47, A/New Caledonia/20/99, A/Mal/302/54, A/Denver/1/57, A/NWS 33, A/Weiss/43, A/Solomon Islands 3/2006); A/H3 strains (A/Alice, A/MRC2, A/Aichi/2/68, A/Brisbane/10/07, A/HongKong/8/68, A/Port Chalmers/1/73, A/Victoria/3/75, A/Wisconsin/67/05); and influenza A/H1N1 strains of swine origin (A/Swine/1976/33, A/Swine Iowa/15/30, A/New Jersey/8/76). All strains tested were detected as positive for influenza A, while only human 2009 H1N1 strains were detected as positive for 2009 H1N1; tested swine origin H1N1 influenza viruses were detected as negative for 2009 H1N1. Compared with other EUA assays (e.g. Prodesse ProFlu-ST Influenza A Assay), which detect swine origin H1N1 viruses as positive,[20] this indicates that the Liat assay is highly specific for 2009 H1N1 viruses.

Cross-reactivity

Cross-reactivity tests the assay's performance for detecting non-target pathogens as negative. Cross-reactivity was tested using medically relevant concentrations of different human pathogens. The test panel comprised 25 viruses and 16 bacteria. Bacteria were tested at

Figure 2: Crossing Threshold Correlation between Liat Influenza A/2009 H1N1 Assay and Comparator Emergency Use Authorization Assays



Crossing threshold (Ct) for influenza A and 2009 H1 targets of the Liat assay are plotted against that of the Focus Diagnostic Influenza A H1N1 (2009) Realtime Polymerase Chain Reaction (RT-PCR) (A, B), Centers for Disease Control and Prevention (CDC) Swine Influenza Virus RT-PCR Panel (C, D) and Roche Realtime Ready Influenza A/H1N1 Detection Set (E). Formula of linear regression line and correlation coefficient (R^2) are shown in each plot. The data demonstrates that the Liat assay is comparable to other Emergency Use Authorization assays.

10^6 to 10^8 cfu/ml or 10^4 $TCID_{50}$/ml (Chlamydia). Viruses were tested at 10^3 to 10^{10} $TCID_{50}$/ml.[21] Bacteria tested include *Bordetella pertussis*, *Chlamydia pneumoniae*, *Corynebactrium*, *Escherichia coli*, *Haemophilus influenzae*, *Lactobacillus sp.*, *Legionella pneumophila*, *Moraxelaa Catarrhalis*, *Neisseria meningitides*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, *Streptococcus pyogens* and *Streptococcus salivarus*. Viruses tested include: adenovirus type 1 and 7; human coronavirus 229E and OC43; enterovirus; human parainfluenza type 1, 2 and 3; measles; human metapneumovirus; mumps virus; respiratory syntial virus type B; rhinovirus type 1A; cytomegalovirus; epstein barr virus, and 10 influenza B viruses (B/Allen/45, B/Florida/7/04, B/GL/1739/54, B/Mass/3/66, B/Taiwan/2/62, B/Maryland/1/59, B/HongKong/5/72, B/Lee/40, B/Malaysia/2506/04, B/Florida/04/06). All tested viruses and bacteria were detected as negative for influenza A and 2009 H1N1. The results demonstrate no cross-reactivity to the tested human pathogens.

Clinical Performance

The performance of the Liat Influenza A/2009 H1N1 Assay was further validated on 226 clinical samples. Of these, 146 samples were collected during the 2009–2010 influenza season; comparator testing for these samples was performed by the TriCore Reference Laboratory (Albuquerque, NM) or New York State Department of Health (Albany, NY) using one of three EUA assays: Focus Diagnostic Influenza A H1N1 (2009) RT-PCR, CDC Swine Influenza Virus RT-PCR Panel or Roche Realtime Ready Influenza A/H1N1 Detection Set. To test specificity for non-2009 H1N1 samples, 80 samples collected during the 2008–2009 flu season prior to the emergence of the 2009 H1N1 were tested; comparator testing for these samples was performed by viral culture and direct fluorescence antibody (DFA) staining at Viromed Laboratories, Laboratory Corporation of America (Minnetonka, Minnesota), or using the RT-PCR method at New York State Department of Health. *Table 2* shows the distribution of samples and comparator test methods. All clinical samples were tested using the Liat Influenza A/2009 H1N1 Assay between March and May 2010. *Table 3* summarises the results of the clinical sample study and performance of the Liat Influenza A/2009 H1N1 Assay against the comparator tests. Per cent positive and negative agreement were

Table 3: Clinical Performance of the Liat Influenza A/2009 H1N1 Assay for 2009 H1 and Influenza A Target Compared with Comparator Tests

2009 H1			
Liat Influenza A/H1N1 Assay	Comparator Tests		
	Positive	Negative	Total
Positive	65	0	65
Negative	0	161	161
Total	65	161	226
	Positive	Negative	
agreement (%)	100.0	100.0	
95% CI lower (%)	94.4	97.7	
95% CI upper (%)	100.0	100.0	

Influenza A			
Liat Influenza A/H1N1 Assay	Comparator Tests		
	Positive	Negative	Total
Positive	126	0	126
Negative	0	100	100
Total	126	100	226
	Positive	Negative	
agreement (%)	100.0	100.0	
95% CI lower (%)	97.0	96.3	
95% CI upper (%)	100.0	100.0	

The Liat assay demonstrated 100% positive and negative agreement for both influenza A and 2009 H1 detection, with lower 95% confidence interval (CI) exceeding 94.4%. Percentage agreement was calculated using the score method.

calculated using the score method.[22] In the 226 clinical samples tested, the Liat Influenza A/H1N1 Assay demonstrated 100% positive and negative per cent agreement for both influenza A and 2009 H1N1 detection, with lower 95% confidence interval (CI) >94.4% in all cases. These data indicate excellent agreement with the comparator tests for positive and negative clinical samples.

The RT-PCR crossing threshold (Ct) values of the Liat Influenza A/2009 H1N1 Assay for these clinical samples were further compared with that of comparator EUA RT-PCR assays. The Liat assay and the comparator EUA assays are qualitative tests. As such, the positive or negative detection result is the determining factor in clinical performance. However, as the Ct is proportional to the order of magnitude of viral load tested, this provides a fine-scale comparison of these assays. *Figure 2* shows the comparison between the Ct for influenza A and 2009 H1 targets of the Liat assay, and Focus assay (panels A and B), CDC assay (panels C and D) or Roche assay (panel E). The slope of linear regression line was between 0.85 and 1.11 (R^2=0.75–0.86) for the comparison between the Liat assay and Focus or CDC assays, suggesting significant correlation. Comparison between Liat and Roche assays had a slope of 0.43 (R^2=0.48), indicating that the correlation was positive but not as strong as that with the other assays. This may be caused by different RT-PCR efficiency due to different reagents, or primers and probes used by each assay. These differences also did not affect the qualitative detection results. Overall, the clinical sample test data demonstrated substantially equivalent assay performance between Liat Influenza A/2009 H1N1 Assay and the other EUA RT-PCR assays.

Discussion

The results of the Liat assay strongly indicated that the system can be used in near-patient settings to provide high-performance nucleic acid test results with the simplicity and speed of current lateral flow immunoassays. The Liat system's ease-of-use, full automation, internal controls and integrated self-corrective and error diagnostic features enable non-specialised healthcare workers to perform testing reliably in near-patient settings. Because all the reagents are self-contained in the Liat tube and all sample processing is performed in the Liat Tube, the system is truly closed, thus avoiding cross-contamination and biohazard risks and allowing testing to be performed outside of high-complexity laboratories. The Liat Influenza A/2009 H1N1 Assay is one of only two EUA 2009 H1N1 nucleic acid tests that received moderate-complexity rating from the US FDA. This allows the test to be used in the larger number of moderate-complexity laboratories, including those in the majority of hospitals and some clinics, and thus increases access to such advanced molecular diagnostics.

With a turnaround time of 26 minutes the Liat Influenza A/2009 H1N1 Assay was also the fastest nucleic acid test that received US FDA EUA for 2009 H1N1 detection; to the best of our knowledge, this is the fastest nucleic acid amplification-based test in the industry to date. Conventional nucleic acid tests require several hours to perform, not including the wait time required to fill test batches, which can extend the turnaround time to one or more days. Even the next fastest EUA nucleic acid test for 2009 H1N1 takes about double the time of the Liat assay to yield a result. Indeed, the Liat assay's 26 minute turnaround time approaches that of point-of-care lateral flow immunoassays and allows testing to be done while the patient is waiting. Combined with the high sensitivity and specificity, this rapid time-to-result enables reliable results to be received immediately for more efficient therapeutic intervention and pandemic response. By eliminating batch reagent waste, hands-on time, specialised testing facilities and sample transport, as well as by reducing the training required to perform tests, the Liat assay is further expected to reduce the overall cost of testing.

Conclusion

The Liat Influenza A/2009 H1N1 Assay has demonstrated 100% positive and negative agreement with other EUA assays from Roche, Focus Diagnostics and the US CDC (as well as with viral culture) in 226 clinical samples tested. Analytical studies further demonstrated equivalent or better LOD compared with such assays, reactivity against all 2009 H1N1 and seasonal influenza A strains tested, and no cross-reactivity with all non-target pathogens tested. While maintaining equivalent or better assay performance as other EUA assays, the Liat assay takes approximately one-seventh of the time required (26 minutes from sample to result), eliminates all manual processing and data interpretation, and can allow the test to be performed in a large number of near-patient moderate-complexity laboratories. To the best of our knowledge, this is the first time that a nucleic acid test has been brought to similar speed and simplicity as a point-of-care immunoassay. Indeed, the performance data for H1N1 has proven the capabilities of the Liat systems, and the development of assays against other targets is expected to bring such benefits to a wide range of clinical diagnostic needs. Such capabilities will enable wider access to nucleic acid testing, rapid diagnostics for timely therapeutic intervention, more efficient laboratory workflow, convenience for the physician and better patient outcomes overall. ■

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