Protocols

Analytical evaluation of the microarray-based FluChip-8G Influenza A + B Assay

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ABSTRACT

Background: Influenza causes a significant annual disease burden, with characterization of the infecting virus important in clinical and public health settings. Rapid immunoassays are fast but insensitive, whereas real-time RT-PCR is sensitive but susceptible to genetic mutations and often requires multiple serial assays. The FluChip-8G Influenza A + B Assay provides type and subtype/lineage identification of influenza A and B, including non-seasonal A viruses, in a single microarray-based assay with same day turnaround time.

Objective: To evaluate key analytical performance characteristics of the FluChip-8G Influenza A + B Assay.

Study design: Analytical sensitivity, cross-reactivity, and multi-site reproducibility were evaluated.

Results: The limit of detection (LOD) for the FluChip-8G influenza A + B Assay ranged from 5.8×10^2–1.5×10^5 genome copies/mL, with most samples $\sim 2 \times 10^3$ genome copies/mL (~ 160 genome copies/reaction). Fifty two (52) additional strains were correctly identified near the LOD, demonstrating robust reactivity. Two variant viruses (H1N1v and H3N2v) resulted in dual identification as both "non-seasonal influenza A" and A/H1N1pdm09. No reproducible cross-reactivity was observed for the 34 organisms tested, however, challenges with internal control inhibition due to crude growth matrix were observed. Lastly, samples tested near the LOD showed high reproducibility (97.0% (95% CI 94.7–98.7)) regardless of operator, site, reagent lot, or testing day.

Conclusion: The FluChip-8G Influenza A + B Assay is an effective new method for detecting and identifying both seasonal and non-seasonal influenza viruses, as revealed by good sensitivity and robust reactivity to 52 unique strains of influenza virus. In addition, the lack of cross-reactivity to non-influenza pathogens and high lab-to-lab reproducibility highlight the analytical performance of the assay as an alternative to real-time RT-PCR and sequencing-based assays. Clinical validation of the technology in a multi-site clinical study is the subject of a separate investigation.

1. Background

Influenza A and B virus infections cause a significant annual disease burden, with the 2017–2018 season causing an estimated 48.8 million illnesses, 959,000 hospitalizations, and 79,400 deaths in the United States (Centers for Disease Control and Prevention, 2019). While detection is important clinically to enable efficient administration of antivirals and curb unnecessary administration of antibiotics, influenza requires robust ongoing annual surveillance of circulating strains to both monitor for antigenic drift and shift and guide annual vaccine strain selection (Jester et al., 2018).

Rapid immunoassays for influenza often suffer from relatively poor sensitivity (Cho et al., 2013; Chan et al., 2007; Talbot et al., 2010; Gavin and Thomson, 2004). Molecular assays based on real-time RT-PCR are typically performed by clinical and public health laboratories and provide excellent sensitivity. However, real-time RT-PCR-based

tests typically amplify short portions of an internal gene segment for typing, and hemagglutinin (HA) and neuraminidase (NA) gene segments for subtyping. Mutations in these targeted regions due to antigenic drift have caused decreased assay performance or failure (Stellrecht, 2018; Huzly et al., 2016; Yang et al., 2014). In addition, many real-time RT-PCR assays require numerous singleplex assays run serially to characterize a specimen, increasing complexity and time to result for HA and NA subtyping. Planar substrate and bead-based microarray-based assays for influenza are also commercially available to type and often subtype influenza A as part of a multi-pathogen respiratory panel. These assays typically rely on a single amplified target as an indicator of the presence of a pathogen. While numerous microarray-based assays utilizing multiple probes per target for the detection of human and animal-origin influenza have appeared (Mukherjee and Chakrabarti, 2012; Heydarov et al., 2017; Paulin et al., 2014; Shi et al., 2014; Ryabinin et al., 2011; Gall et al., 2009; Li et al., 2009), none are broadly commercially available. Next generation sequencing of influenza viruses provides the ability for complete characterization and is now routine in the three National Influenza Surveillance Reference Centers and at the CDC. However, next generation sequencing is not yet common for broader influenza surveillance or clinical use in part due to limited availability, cost, and complexity of data analysis (Jester et al., 2018; Balloux et al., 2018; Hampson et al., 2017).

The FluChip-8G Influenza A + B Assay (InDevR, Inc., Boulder, CO) was developed to address a need in influenza diagnosis and pandemic preparedness by providing same day influenza typing and subtype/lineage identification for clinicians and public health scientists. Designed to detect and distinguish seasonal influenza A/H1N1pdm09, A/H3N2, and A/"non-seasonal" (defined as all other influenza A viruses), B/Yamagata, and B/Victoria viruses, the assay utilizes a high information content microarray coupled with an automated pattern recognition-based software algorithm for data interpretation.

1.1. Objectives

The aim of this study was to evaluate key analytical performance metrics for the FluChip-8G Influenza A + B Assay, including analytical sensitivity (LOD), analytical reactivity (inclusivity) and specificity (cross-reactivity with non-influenza pathogens), and multi-site reproducibility.

2. Study design

2.1. FluChip-8G Influenza A + B Assay technology

The FluChip-8G Influenza A + B Assay (InDevR Inc., Boulder, CO) is a multiplexed RT-PCR and microarray-based assay that detects nucleic acid from seasonal and non-seasonal influenza A and B viruses. The assay employs a universal priming mixture to amplify several full length gene segments of influenza A and B viruses, followed by hybridization and detection using a DNA microarray containing 458 short oligonucleotide capture sequences that represent ∼25% of the entire influenza genome to provide comprehensive information. A pattern recognition-based approach is then used in which signals from all microarray capture sequences are utilized to characterize the virus(es) present.

The FluChip-8G Influenza A + B Assay was executed according to the manufacturer's instructions, and involves three main steps: 1) nucleic acid extraction, 2) multiplexed one-step RT-PCR amplification, and 3) capture and detection of fragmented amplicons on a DNA microarray. In brief, the QIAamp MinElute Virus Spin Kit (Qiagen, Inc.) was used for nucleic acid extraction. Extracted nucleic acid was amplified using the FluChip-8G Amplification Reagents (InDevR, Inc.) which amplify full-length influenza A gene segments HA, NA, M, nonstructural (NS), and nucleoprotein (NP), full length influenza B HA and NA gene segments, and a portion of the eukaryotic 18S rRNA gene as an internal control. During amplification, biotin-16-aminoallyl-2' dUTP is incorporated to enable downstream labeling by a streptavidin-coupled fluorophore. Post-amplified PCR products were fragmented at 95 °C for 10 min prior to microarray hybridization. After hybridization, the microarray was labeled and analyzed using the fluorescence-based FluChip-8G Imaging System (InDevR, Inc.).

2.2. Preparation of contrived samples

All samples evaluated consisted of influenza-spiked nasal specimens. Nasal specimens stabilized in universal transport medium that were tested to be negative for influenza virus by the GenMark eSensor RVP kit were purchased from Discovery Life Sciences. All influenza negative clinical specimens received were screened by the CDC human influenza virus real-time RT-PCR Diagnostic Panel to further confirm influenza negativity and subsequently combined to create a pooled clinical matrix (PCM) for the preparation of influenza-spiked samples used throughout the study.

The A/H1N1pdm09, A/H3N2, B/Victoria, and B/Yamagata viruses were obtained from either Zeptometrix or the CDC repositories. All other influenza viruses were sourced from Zeptometrix, International Reagent Resource, BEI resources, the CDC repositories, or St. Jude Children's Research Hospital. Samples were received with a certificate of analysis and/or sequence information identifying HA and NA subtype. Whenever possible, influenza-spiked samples were prepared by spiking intact virus into PCM prior to evaluation. However, several strains were utilized as extracted RNA due to biosafety concerns or unavailability of intact virus. In these cases, viral RNA was spiked into PCM along with the protease and lysis buffer to stabilize the naked viral RNA prior to re-extraction of the influenza-spiked sample.

2.3. Analytical sensitivity and inclusivity

The analytical sensitivity of the FluChip-8G Influenza A + B Assay was assessed using 18 unique virus strains representing each targeted group: (10) non-seasonal A, (2) A/H3N2, (2) A/H1N1pdm09, (2) B/Yamagata, and (2) B/Victoria (Table 1). Range-finding studies were conducted to determine the assay cutoff and approximate LOD for each strain, followed by analysis of at least 20 replicates at the approximate LOD to determine the concentration resulting in a ≥ 95% positivity rate. Inclusivity/analytical reactivity was assessed using an additional 52 influenza-spiked samples containing virus strains representing temporal, geographic, and species of origin diversity not included in the LOD testing: (20) non-seasonal A, (10) A/H3N2, (11) A/H1N1pdm09, (5) B/Yamagata, and (6) B/Victoria. Inclusivity testing was conducted at concentrations near the LOD with each sample analyzed in triplicate (Table 2).

2.4. Cross-reactivity

Cross-reactivity of the assay with bacteria and non-influenza viruses was evaluated using a panel of 34 non-influenza pathogens (19 bacteria, 15 viruses) (Table 3). Contrived samples were prepared by spiking the bacteria and viruses into PCM at concentrations $\geq 1 \times 10^6$ cfu/mL for bacteria and $\geq 1 \times 10^5$ TCID$_{50}$/mL for viruses whenever possible. Each pathogen was extracted once and the extracted nucleic acid tested in triplicate. Additional replicate analyses were performed for any false positive results to evaluate consistency of assay results in the presence of potentially cross-reactive pathogens.

2.5. Multi-site reproducibility

The reproducibility of the FluChip-8G Influenza A + B Assay was assessed using a blinded panel of 45 contrived samples comprised of one representative strain of each of the 5 influenza target virus groups. Each strain was analyzed at three different concentrations (∼0.1x,

Table 1
FluChip-8G Influenza A + B Assay limit of detection (LOD).

Strain Tested	Positivity		95% CI (LCL-UCL)	LOD (copies/mL)
	#/total	%		
A/H1N1pdm09 strains				
A/New York/01/2009	25/25	100%	86.7–100	1.4×10^3
A/California/07/2009	20/20	100%	83.9–100	1.8×10^3
A/H3N2 strains				
A/Perth/16/2009	24/25	96%	80.5–99.3	1.4×10^3
A/Victoria/361/2011	25/25	100%	83.9–100	1.2×10^3
B/Victoria lineage strains				
B/Brisbane/60/2008	24/25	96%	80.5–99.3	5.8×10^2
B/Florida/02/2006	20/20	100%	83.9–100	1.3×10^3
B/Yamagata lineage strains				
B/Wisconsin/01/2010	25/25	100%	86.7–100	6.3×10^2
B/Phuket/3073/2013	20/20	100%	83.9–100	1.7×10^3
A/Non-seasonal strains				
A/New Caledonia/20/1999 (H1N1 pre 2009)	20/20	100%	83.9–100	5.6×10^3
A/Anhui/01/2005 (H5N1)	20/20	100%	83.9–100	3.2×10^3
A/mule duck/Bulgaria/328/2011 (H5N8)	20/20	100%	83.9–100	4.9×10^3
A/ruddy turnstone/New Jersey/ AI11-1678/2011 (H7N7)	20/20	100%	83.9–100	1.2×10^3
A/mallard/Alberta/177/2004 (H7N9)	20/20	100%	83.9–100	5.8×10^2
A/mule duck/Bulgaria/237/2011 (H5N2)	20/20	100%	83.9–100	1.5×10^3
A/shorebird/Delaware Bay/127/ 2003 (H9N2)	20/20	100%	83.9–100	7.7×10^2
A/swine/Illinois/4L013/2015 (H1N1v)	24/25	96%	80.5–99.3	1.8×10^5
A/Indiana/08/2011 (H3N2v)	19/20	95%	76.4–99.1	2.9×10^3
A/blue-winged teal/Iowa/ 10OS2411/2010 (H3N8)	20/20	100%	83.9–100	8.7×10^2

~1x, and ~3x LOD) with each concentration of each strain analyzed in triplicate. Samples were tested at InDevR and two other independent labs, using 2 different operators at each site (6 operators total). Two unique lots of reagents were utilized and evenly divided between the 3 sites.

2.6. Statistical analysis

Viruses were considered detected if the expected assay output was obtained. For each study, total percent agreement with the expected result and associated 95% confidence intervals (Wilson score interval) were calculated.

3. Results

3.1. Analytical sensitivity/LOD

Limits of detection (LOD), at which ≥ 95% of replicates tested produced the expected result, are presented in Table 1. Analytical sensitivity ranged from 5.8×10^2 and 1.5×10^5 approximate genome copies/mL.

All 52 strains analyzed to demonstrate analytical reactivity were correctly identified in all three replicates at the concentrations tested (Table 2).

3.2. Cross-reactivity

No consistent cross-reactivity was observed for any non-influenza pathogen tested (Table 3). During the course of testing, six bacteria (*M. catarrhalis*, *S. pyogenes*, *S. salivarius*, *P. aeruginosa* (strain Shr42), *M. pneumoniae* and *N. meningitidis*) initially resulted in inhibition of the internal control likely due to the presence of crude growth medium. This inhibition was resolved either by further dilution of the matrix, or

through use of purified organisms. Three (3) organisms resulted in a false positive for influenza in 1 of the replicates tested, with the other 2 replicates producing the expected "influenza not detected" result. Additional replicate analysis demonstrated that these false positive results were not reproducible: only 2/31 replicates analyzed for *P. aeruginosa* (strain Shr42), 1/6 for *M. pneumoniae*, and 1/6 *N. meningitidis*, respectively, resulted in false positive results.

3.3. Multi-site reproducibility

Site-to-site assay reproducibility is presented in Table 4. Overall positive agreement of 99.6% (95% CI of 97.9–99.9%) was achieved for samples analyzed at ~3x LOD. Overall positive agreement of 97% (95% CI of 94.7–98.7) was achieved for samples analyzed at ~1x LOD. Samples tested at ~0.1x LOD (data not shown) had lower overall reproducibility, as expected, with percent positivity of 22.6% (95% CI of 18.0–27.9). As with all PCR-based tests, concentrations below the LOD may be detected, but results are not reproducible.

Of the 810 contrived samples analyzed, 13 (1.6%) were misidentified as a type or subtype/lineage not present in the sample. Nine (9) of the samples resulting in misidentification were at ~0.1x LOD and four (4) were at ~1x LOD. Instances of sample misidentification occurred at all three sites. Two (2) out of the 810 samples analyzed (0.2%) resulted in assay control failures.

4. Discussion

The FluChip-8G Influenza A + B assay was capable of detecting influenza RNA at ~2×10^3 genome copies/mL for all strains except the H1N1v tested (Table 1). This translates to a detection sensitivity of ~160 genome copies/reaction. While the H1N1v virus tested (A/ swine/Illinois/4L013/2015) was consistently identified correctly as influenza A, it was often identified as both A/non-seasonal and misidentified as an A/H1N1pdm09 virus. The LOD for this strain was therefore based on the concentration above which the results included the correct call of A/non-seasonal. Below the LOD, the H1N1v samples were misidentified as A/H1N1pdm09 without the corresponding A/ non-seasonal result. The H3N2v virus strain (A/Indiana/08/2011) resulted in correct identification as non-seasonal influenza A above the LOD, but was misidentified at lower concentrations as an A/ H1N1pdm09. These misidentifications are likely due to the genetic similarity of H1N1v and H3N2v strains to the seasonally circulating H1N1pdm09 virus. The H1N1pdm09 virus has continually reassorted with swine influenza A viruses, generating diverse reassortant genotypes (Nelson et al., 2016). Specifically, the H1N1v for which the LOD was determined, A/swine/Illinois/4L013/2015, shares the M and NP gene segments with H1N1pdm09 strains, and the HA and NS gene segments have > 90% sequence homology. The H3N2v strain (A/Indiana/08/2011) shares the M gene with H1N1pdm09 strains, and the NP and NS gene segments also have > 90% sequence homology. Given that HA, NA, M, NP, and NS are the gene segments targeted by the FluChip-8G assay, it is not surprising that these viruses are mis-identified at low concentrations. In the event of an H1N1v or H3N2v infection, we note that the specimen would still be identified as influenza positive, enabling appropriate treatment. In addition, specimens with high enough H1N1v or H3N2v titer would produce a dual infection result that would prompt reanalysis and still enable appropriate follow-up by public health authorities.

All additional virus strains tested near the LOD were correctly identified (Table 2), demonstrating broad analytical reactivity. Of particular note is the correct identification of 9 non-seasonal A subtypes (H1N1sw, H1N8, H2N2, H3N2sw, H3N9, H4N6, H6N4 and H11N2) *not* represented in the LOD study. The accurate detection of these subtypes indicates the broad detectability of non-seasonal influenza A viruses by the assay. We also note that additional H1N1v and H3N2v strains included in analytical reactivity testing were correctly identified at the

Table 2

FluChip-8G Influenza A + B Assay detection of 52 additional strains, demonstrating analytical reactivity.

Virus Designation	Target Virus Group/ subtype	copies/mL tested
A/South Carolina/18/2009	Seasonal H1N1 (2009)	2.5×10^3
A/Canada/6294/2009		1.3×10^3
A/Netherlands/2290/2009		5.0×10^3
A/New York/18/2009		9.9×10^2
A/Utah/20/2009		1.0×10^4
A/Massachusetts/15/2013		7.9×10^3
A/Mexico/4108/2009		1.9×10^3
A/New Hampshire/02/2010		3.2×10^3
A/California/04/2009		4.7×10^3
A/Dominican Republic/7293/2013		3.9×10^3
A/Michigan/45/2015		6.5×10^3
A/Stockholm/6/2014	Seasonal H3N2	2.4×10^3
A/South Australia/55/2014		2.2×10^2
A/Switzerland/9715293/2013		5.7×10^2
A/Norway/466/2014		5.3×10^2
A/Texas/71/2007		4.2×10^3
A/Uruguay/716/2007		5.2×10^3
A/Texas/50/2012		3.9×10^3
A/Santiago/7981/2006		2.9×10^3
A/Wisconsin/67/2005		5.3×10^2
A/Hong Kong/4801/2014		2.1×10^3
B/Nevada/03/2011	B/Victoria	1.3×10^3
B/Malaysia/2506/2004		1.7×10^3
B/New Jersey/01/2012		2.6×10^3
B/Victoria/304/2006		4.7×10^3
B/Hong Kong/330/2001		3.4×10^3
B/Texas/02/2013		1.6×10^3
B/Texas/06/2011	B/Yamagata	3.6×10^3
B/Pennsylvania/07/2007		3.8×10^3
B/Massachusetts/02/2012		5.3×10^3
B/Brisbane/3/2007		2.8×10^3
B/Florida/07/2004		1.2×10^3
A/Fujian Gulou/1896/2009	H1N1 pre09	3.4×10^3
A/Swine/1976/1931	H1N1sw	6.9×10^3
A/Swine/IL/4L036/2015	H1N1v	6.7×10^4
A/Red Knot/Delaware Bay/240/1994	H1N8	4.5×10^3
A/Chicken/NY/SG-00425/2004	H2N2	5.6×10^3
A/Swine/Ohio/11SW87/2011	H3N2sw	7.9×10^3
A/Minnesota/11/2010	H3N2v	1.0×10^4
A/Blue-Winged Teal/Alberta/ 221/1978	H3N8	1.6×10^3
A/Camel/Mongolia/335/2012	H3N8	5.6×10^3
A/Mallard/Alberta/54/1993	H3N9	2.1×10^3
A/Blue-Winged Teal/IL/10OS1563/2010	H4N6	5.6×10^3
A/Goose/Yunnan/5539/2005	H5N1	4.5×10^3
A/chicken/Vietnam/NCVD-016/2008	H5N1	7.9×10^3
A/Northern Pintail/CA/44242-758/2006	H5N2	2.6×10^4
A/Duck/England/36254/2014	H5N8	7.3×10^3
A/Mallard/Alberta/58/1989	H6N4	2.1×10^3
A/Italy/3/2013	H7N7	1.7×10^4
A/Hong Kong/5942/2013	H7N9	6.9×10^3
A/Turkey/Wisconsin/1/1966	H9N2	3.0×10^3
A/Laughing Gull/Delaware Bay/94/1995	H11N2	1.4×10^4

(The A/non-seasonal group spans rows A/Fujian Gulou/1896/2009 through A/Laughing Gull/Delaware Bay/94/1995.)

concentrations tested.

No consistent cross-reactivity with any of the non-influenza pathogens tested was observed (Table 3). As mentioned, six (6) of the thirty-four (34) tested bacteria and viruses did result in at least one internal control failure at the initial concentration analyzed. As these microorganisms were not purified, it was hypothesized that the complex crude bacterial growth medium they were supplied in caused assay inhibition resulting in internal control failures. To further investigate whether the internal control failures were due to the growth medium or to the organism itself, the microorganisms were subsequently diluted and reanalyzed. In four (4) cases (*M. catarrhalis*, *S. pyogenes*, *S. salivarius*, and *P. aeruginosa* (strain Shr42)) the cross-reactivity was resolved, indicating the growth medium was the likely cause. Internal control failures recurred in samples containing *M. pneumoniae* and *N. meningitidis* even after dilution. Purified samples of these organisms were obtained, and upon reanalysis all remaining internal control failures were resolved. As indicated above, the bacterial growth medium would not be expected in a clinical or surveillance setting for

which the assay is intended.

The FluChip-8G Influenza A + B Assay demonstrated good precision in a multi-site reproducibility study with robust identification of all targets observed (Table 4). The expected assay results were obtained for 98.5% (95% CI of 97.1–99.2) of samples run at or above the LOD regardless of operator, site, testing day, and reagent lot. Below the LOD, a positive influenza result was obtained in only 22.6% (95% CI of 18.0–27.9) of samples run below the LOD. This increased variability is expected for concentrations below the assay LOD due to the stochastic nature of PCR-based amplification at low input copy number.

Overall, the excellent analytical performance of the FluChip-8G Influenza A + B Assay highlights its utility in the typing and subtyping/ lineage determination of seasonally circulating A and B viruses, and positive detection of 'non-seasonal' influenza viruses in a single, multiplexed assay. If challenged with an infection of a novel or emerging influenza A virus strain, most available assays would produce an influenza A "unsubtypeable" result that would require reflex testing to an alternative assay with the capability to screen for high priority non-

Table 3

Bacteria and viruses evaluated for potential cross-reactivity with the FluChip-8G Influenza A + B Assay. Strains are listed in parentheses.

Virus	Concentration tested		Bacterium	Concentration tested	
Adenovirus (Adenoid 71)	8.0×10^6	$TCID_{50}/mL$	*B. pertussis* (A639)	3.8×10^9	cfu/mL
Adenovirus (Type 7A, species B)	3.0×10^7	$TCID_{50}/mL$	*C. pneumoniae* (AR-39)	8.0×10^5	$TCID_{50}/mL$
Coronavirus (229E)	2.5×10^5	$TCID_{50}/mL$	*C. diphtheriae* (48255)	2.3×10^6	cfu/mL
Coronavirus (OC43)	1.4×10^5	$TCID_{50}/mL$	*E. coli* (H10407 (O78:H11)	4.7×10^9	cfu/mL
Cytomegalovirus (ATCC-2011-8)	1.2×10^5	$TCID_{50}/mL$	*H. influenza* (type B; MinnA)	2.7×10^8	cfu/mL
Enterovirus (HEV-71, MP4)	8.0×10^5	$TCID_{50}/mL$	*L. acidophilus* (Scav)	1.3×10^8	cfu/mL
Epstein Barr Virus (P-3)	2.2×10^8	$TCID_{50}/mL$	*L. pneumophila* (Philadelphia)	9.0×10^9	cfu/mL
Human parainfluenza (Type 1)	9.5×10^6	$TCID_{50}/mL$	*M. catarrhalis* (Ne 11)	6.5×10^6	cfu/mL
Human parainfluenza (Type 2, Greer)	4.5×10^5	$TCID_{50}/mL$	*M. tuberculosis* (H37Ra-1)	7.0×10^6	cfu/mL
Human parainfluenza (Type 3)	2.5×10^5	$TCID_{50}/mL$	*M. pneumoniae* (FH (Eaton Agent))- purified	1.0×10^6	ccu/mL
Measles	1.3×10^5	$TCID_{50}/mL$	*N. meningitidis* (M1027 [NCTC 10025])- purified	1.0×10^6	cfu/mL
Human metapneumovirus (IA10-2003)	1.3×10^5	$TCID_{50}/mL$	*N. sicca* (6b)	1.8×10^8	cfu/mL
Mumps virus (Isolate 1)	5.0×10^5	$TCID_{50}/mL$	*P. aeruginosa* (Shr42)	1.0×10^6	cfu/mL
Human respiratory syncytial virus (CH93(18)-18)	1.3×10^5	$TCID_{50}/mL$	*P. aeruginosa* (clinical isolate)-purified	1.0×10^6	cfu/mL
Rhinovirus (Type 1A)	7.0×10^4	$TCID_{50}/mL$	*S. aureus* (NCTC 8530 [S11])	7.5×10^8	cfu/mL
			S. epidermidis (12333)	7.5×10^8	cfu/mL
			S. pneumoniae (GA17457)	3.4×10^7	cfu/mL
			S. pyogenes (MGAS9890)	8.5×10^6	cfu/mL
			S. salivarius (Z127)	3.8×10^6	cfu/mL

Table 4

Multi-site reproducibility of the FluChip-8 G Influenza A + B Assay.

Target Group		Site A			Site B			Site C			Combined		
		Agreement			Agreement			Agreement			Agreement		
		n/total	%	95% CI	n/total	%	95% CI	n/total	%	95% CI	n/total	%	95% CI
A/H1N1pdm09	3x LOD	18/18	100%	82.4 - 100	18/18	100%	82.4 - 100	18/18	100%	82.4 - 100	54/54	100%	93.4 - 100
	1x LOD	18/18	100%	82.4 - 100	14/18	78%	54.8 - 91.0	17/18	94%	74.2 - 99.0	49/54	91%	80.1 - 96.0
A/H3N2	3x LOD	18/18	100%	82.4 - 100	18/18	100%	82.4 - 100	18/18	100%	82.4 - 100	54/54	100%	93.4 - 100
	1x LOD	18/18	100%	82.4 - 100	18/18	100%	82.4 - 100	18/18	100%	82.4 - 100	54/54	100%	93.4 - 100
B/Yamagata	3x LOD	18/18	100%	82.4 - 100	18/18	100%	82.4 - 100	18/18	100%	82.4 - 100	54/54	100%	93.4 - 100
	1x LOD	18/18	100%	82.4 - 100	17/18	94%	74.2 - 99.0	18/18	100%	82.4 - 100	53/54	98%	90.2 - 99.7
B/Victoria	3x LOD	18/18	100%	82.4 - 100	18/18	100%	82.4 - 100	18/18	100%	82.4 - 100	54/54	100%	93.4 - 100
	1x LOD	17/18	94%	74.2 - 99.0	18/18	100%	82.4 - 100	18/18	100%	82.4 - 100	53/54	98%	90.2 - 99.7
A/non-seasonal	3x LOD	18/18	100%	82.4 - 100	18/18	100%	82.4 - 100	17/18	94%	74.2 - 99.0	53/54	98%	90.2 - 99.7
	1x LOD	18/18	100%	82.4 - 100	18/18	100%	82.4 - 100	18/18	100%	82.4 - 100	54/54	100%	93.4 - 100

seasonal A viruses such as H5N1 or H7N9. The ability to detect and positively identify a wide variety of novel or emerging influenza viruses as "non-seasonal" influenza A in the same assay in which seasonally circulating influenza viruses are characterized is therefore unique compared to other available influenza molecular diagnostic assays. In addition, the full gene segment amplification approach combined with pattern-recognition based data analysis allows for rapid updates to the software algorithm as new viruses emerge, without requiring changes to the assay procedure. We hope the availability of this same day result will better enable rapid and appropriate notification of public health authorities in the case of identification of a potentially pandemic virus to enhance pandemic preparedness efforts. Clinical validation of the technology in a multi-site clinical study is the subject of a separate investigation and will be published separately.

CRediT authorship contribution statement

Amber W. Taylor: Funding acquisition, Conceptualization, Supervision, Methodology, Investigation, Formal analysis, Writing - original draft, Writing - review & editing. **Erica D. Dawson:** Funding acquisition, Project administration, Resources, Conceptualization, Supervision, Methodology, Investigation, Formal analysis, Writing - original draft, Writing - review & editing. **Rebecca H. Blair:** Methodology, Formal analysis, Investigation, Writing - review & editing. **James E. Johnson:** Methodology, Formal analysis, Investigation, Writing - review & editing. **Amelia H. Slinskey:** Methodology, Formal analysis, Investigation, Writing - review & editing. **Andrew W. Smolak:** . **Evan Toth:** Methodology, Formal analysis, Investigation, Writing - review & editing. **Kyle Liikanen:** Methodology, Formal analysis, Investigation, Writing - review & editing. **Robert S. Stoughton:** Methodology, Formal analysis, Investigation, Writing - review & editing. **Catherine Smith:** Methodology, Formal analysis, Investigation, Writing - review & editing. **Sarah Talbot:** Methodology, Formal analysis, Investigation, Writing - review & editing. **Kathy L. Rowlen:** Funding acquisition, Project administration, Resources, Conceptualization, Methodology, Writing - review & editing.

Declaration of Competing Interest

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