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Clinical validation of the FluChip-8G Influenza A + B Assay for influenza type and subtype identification

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ABSTRACT

Background: The FluChip-8G Influenza A + B Assay is a multiplexed influenza RT-PCR and microarray-based assay with same day turnaround time, developed to subtype seasonal A viruses (H1N1pdm2009 and H3N2), distinguish B viruses as Yamagata or Victoria lineage, and is the only FDA cleared assay capable of positive identification of a wide variety of A subtypes as "non-seasonal" A viruses from human nasal specimens.
Objective: To evaluate clinical performance of the FluChip-8G Influenza A + B Assay for detection of seasonal influenza viruses in nasal and nasopharyngeal swab specimens, and to evaluate performance for detection of non-seasonal influenza viruses using contrived samples.
Study Design: For seasonal viruses, a multisite study of the FluChip-8G Influenza A + B Assay using prospectively and retrospectively collected nasal and nasopharyngeal swabs was performed using the FDA-cleared CDC Human Flu Dx Panel as the comparator assay. For non-seasonal viruses, testing was performed at a single site using contrived samples from 100 unique non-seasonal strains representing 41 subtypes.
Results: Sensitivity (95% CI) and specificity (95% CI) for each target group, respectively, from results of 1689 clinical specimens were: seasonal H1N1pdm2009: 96.4% (87.9–99.0), 99.3% (98.8–99.6), seasonal H3N2: 91.8% (87.7–94.7), 99.7% (99.2–99.9), Influenza B Victoria: 100% (94.0–100.0), 99.9% (99.6–100.0), and Influenza B Yamagata: 95.6% (89.2–98.3), 99.9% (99.6–100.0). The sensitivity and specificity from contrived influenza A non-seasonal viruses was determined to be 99.0% (94.6–99.8) and 100% (96.7–100.0).
Conclusion: The FluChip-8G Influenza A + B Assay has robust sensitivity and specificity for detecting and identifying all target virus groups, including non-seasonal influenza A, with same day results.

1. Background

Over the past 8 influenza seasons in the United States, the estimated number of humans infected with influenza ranged from 9.3 million to 49 million per year, with approximately 12,000 to 80,000 deaths occurring each season [1]. There are significant resources dedicated to the ongoing surveillance of influenza to reduce the burden of influenza on public health. While many diagnostic tests for influenza virus exist, the ability to successfully detect and identify influenza viruses as they mutate to evade the human immune system is a challenge faced by many available assays. Of the FDA-cleared influenza molecular diagnostics available at the onset of the 2009 pandemic, only two were capable of subtyping influenza A viruses, and neither was able to subtype the H1N1 pandemic 2009 due to the drastic genotypic and

Abbreviations: CI, confidence interval; HA, hemagglutinin influenza viral RNA segment; H1N1pdm2009, H1N1 pandemic from 2009; FN, false negative; FP, false positive; LCL, lower confidence level; LOD, limit of detection; M, matrix influenza viral RNA segment; NA, neuraminidase influenza viral RNA segment; NS, non-structural influenza viral RNA segment; NP, nucleoprotein influenza viral RNA segment; NPS, nasopharyngeal swabs; RT-PCR, reverse transcriptase polymerase chain reaction; TN, true negative; TP, true positive; UCL, upper confidence level; UTM, universal transport media

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phenotypic changes in the virus [2] compared to the previously circulating H1N1 strains.

Given that many available molecular diagnostic assays would produce an influenza A "unsubtypeable" result for an infection with a novel influenza A virus strain [3–6], the ability to characterize seasonal viruses and simultaneously positively identify a wide variety of animal-origin viruses in human samples as "non-seasonal" in a single assay provides the capability for more timely and complete characterization. In addition, this offers the ability to quickly triage viruses identified as "non-seasonal" for important follow-up testing. The FluChip-8G Influenza A + B Assay (InDevR, Inc., Boulder, CO) was designed to detect and characterize influenza B as well as influenza A viruses in a single assay and is robust to antigenic shift and drift because it utilizes primers that target conserved regions of the influenza genome. The target virus groups identified are seasonal influenza B Victoria and Yamagata lineages, seasonal influenza A viruses H1N1pdm2009 and H3N2, and 'non-seasonal' influenza A viruses, which includes diverse influenza A viruses of zoonotic origin, including subtypes with pandemic potential. The FluChip-8G Influenza A + B Assay utilizes multiplexed RT-PCR that amplifies 5 full length gene segments from influenza A viruses (HA, M, NA, NS, and NP), full length HA and NA from influenza B viruses, and the 18S human gene as an endogenous internal control that is analyzed when influenza is not detected in the sample. Amplified product is detected via hybridization to a microarray representing ~25% of the entire influenza A genome coupled with an automated pattern recognition algorithm.

Analytical performance for the FluChip-8G Influenza A + B Assay was reported in [7]. In summary, 52 seasonal and non-seasonal influenza strains were detected by the assay near the LOD, and no consistent cross-reactivity was observed with the 34 non-influenza pathogens tested. In addition, the assay exhibited high reproducibility of samples near the LOD when tested at 3 different sites by 6 different operators.

This study assessed the clinical performance of the recently 510(k) cleared FluChip-8G Influenza A + B Assay using nasal swabs and NPS specimens containing seasonal influenza viruses or contrived samples for non-seasonal viruses.

1.1. Objectives

The aim of this study was to evaluate the clinical performance of the FluChip-8G Influenza A + B Assay on prospectively and retrospectively collected nasal and nasopharyngeal swabs. Non-seasonal influenza A viruses were not expected to be present in the clinical cohort. Performance of the non-seasonal target virus group was assessed using contrived samples in individual influenza-negative clinical specimens.

2. Study design

2.1. Clinical study design

All specimens tested were either nasal swabs or NPS that were stabilized in Copan UTM and shipped frozen to a reference laboratory.

Prospectively-collected specimens analyzed in the clinical study were thawed and tested with the CDC Human Flu Dx Panel of singleplex assays (the comparator assay) and then blinded and shipped frozen to one of the three participating testing sites (Naval Health Research Center, Naval Medical Center San Diego, and InDevR). Each clinical study testing site performed the FluChip-8G Influenza A + B Assay on approximately one third of the blinded specimens.

Retrospective specimens included in supplemental testing were thawed and analyzed with the CDC Flu Dx Assay, and then blinded and shipped to a single testing site.

After all specimens were tested with the FluChip-8G Influenza A + B Assay, the data were un-blinded and the FluChip-8G Influenza A + B Assay results were compared to results of the comparator assay to assess sensitivity and specificity along with 2-sided 95% confidence intervals.

Following un-blinding and analysis, bi-directional sequencing was performed on specimens for any cases involving 1) discordant results between the comparator assay and the FluChip-8G Influenza A + B Assay, 2) cases in which the FluChip-8G Influenza A + B Assay produced a typing result without an associated subtype, and 3) any results indicating detection of non-seasonal influenza A by the FluChip-8G Influenza A + B Assay.

2.2. FluChip-8G Influenza A + B Assay

All testing was performed using reagents provided in the FluChip-8G Influenza A + B Assay kit (InDevR, Inc., catalog number FC-6101) according to manufacturer instructions. In brief, nucleic acid was extracted from specimens or contrived samples with the QIAamp MinElute Virus Spin Kit (Qiagen, Inc.) using 200 µL of specimen and eluting in 50 µL. Extracted nucleic acid was then amplified using the FluChip-8G amplification reagents and heat fragmented at 95 °C. The fragmented RT-PCR products were then hybridized to the FluChip-8G microarray and the microarray was washed, labeled, washed, and analyzed using the fluorescence-based FluChip-8G Imaging System (InDevR, Inc. catalog number FC-6000).

2.3. Comparator assay

Specimens were analyzed following the FDA-cleared CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel (CDC Human Flu Dx Panel) product insert.

2.4. Prospective clinical study

The clinical performance of the FluChip-8G Influenza A + B Assay was established in a multi-testing site clinical study between June 2017 and July 2018 in the United States. Nasal and nasopharyngeal swab specimens were prospectively collected as part of a routine, annual surveillance protocol from 2013 to 2017 from seven geographically distributed clinical sites across the United States (Table 1). All specimens were frozen prior to testing. Nasal swabs or NPS specimens collected from 1065 subjects were enrolled in this clinical study. Specimens were included in the clinical study if the subject was presenting with a fever ≥ 100.5 °F, influenza like illness, and there was sufficient volume to perform the FluChip-8G Influenza A + B Assay and any potential follow up testing required.

2.5. Supplemental clinical study on prospectively collected specimens

The prevalence of influenza in the primary prospective clinical study was expected to be lower than needed to obtain sufficient numbers of influenza positive specimens to establish FluChip-8G Influenza A + B Assay performance. Therefore, an additional 500 prospectively collected specimens from 2013 to 2017 were included in a supplemental clinical study.

Table 1
Collection sites for specimens in the prospective clinical study.

Collection Site	Location
Branch Health Clinic Yuma/Marine Corps Air Station Yuma	Yuma, AZ
Camp Pendleton (Marine Corps Base and Navy Hospital)	Camp Pendleton, CA
Naval Hospital Great Lakes	Great Lakes, IL
Robert E. Bush Naval Hospital	Twentynine Palms, CA
Naval Training Center, San Diego	San Diego, CA
Naval Medical Center, San Diego	San Diego, CA
Tricare Outpatient Clinic, Kearny Mesa	San Diego, CA

Table 2
Non-seasonal influenza A virus strains included in the study.

Strain	Subtype	Material Input into Extraction	Concentration (approx. genome copies/mL)	Multiple of LOD tested
A/canvasback/Alberta/276/2005	H1N1	whole virus	8.3×10^4	28x
A/redheaded duck/Minnesota/SG-00123/2007	H1N1	whole virus	6.9×10^3	2x
A/mallard/Republic of Georgia/4/2010	H1N1	whole virus	2.5×10^5	84x
A/duck/Alberta/35/1976	H1N1	genomic RNA	2.9×10^4	10x
A/South Dakota/06/2007	H1N1 pre-2009	whole virus	6.4×10^3	2x
A/Florida/03/2006	H1N1 pre-2009	whole virus	6.2×10^5	208x
A/Solomon Islands/3/2006	H1N1 pre-2009	whole virus	3.4×10^3	1x
A/Fukushima/141/2006	H1N1 pre-2009	whole virus	4.5×10^3	2x
A/St. Petersburg/8/2006	H1N1 pre-2009	whole virus	5.5×10^4	18x
A/swine/Ohio/09SW1477/2009	H1N2	whole virus	6.7×10^4	23x
A/swine/Ohio/09SW1484E/2009	H1N2	whole virus	3.7×10^3	1x
A/shorebird/Delaware Bay/211/1994	H1N3	whole virus	2.5×10^5	84x
A/Japan/305/1957	H2N2	genomic RNA	8.9×10^3	3x
A/Duck/Germany/1215/1973	H2N3	genomic RNA	8.7×10^3	3x
A/swine/North Carolina/32760/2007	H3N2sw	genomic RNA	6.3×10^3	2x
A/swine/North Carolina/44897/2009	H3N2sw	genomic RNA	5.4×10^3	2x
A/swine/North Carolina/52796/2006	H3N2sw	genomic RNA	4.0×10^3	1x
A/swine/North Carolina/88708/2000	H3N2sw	genomic RNA	3.7×10^3	1x
A/Indiana/10/2011	H3N2v	whole virus	2.2×10^5	74x
A/Michigan/20/2012	H3N2v	genomic RNA	1.1×10^4	4x
A/Ohio/20/2012	H3N2v	genomic RNA	7.1×10^3	2x
A/Ohio/36/2012	H3N2v	genomic RNA	5.7×10^3	2x
A/Ohio/44/2012	H3N2v	genomic RNA	5.5×10^3	2x
A/blue-winged teal/Illinois/10OS1546/2010	H3N6	whole virus	1.3×10^5	42x
A/redhead/Alberta/192/2002	H3N6	whole virus	4.8×10^3	2x
A/equine/Pennsylvania/1/2007	H3N8	whole virus	1.4×10^3	0.5x
A/equine/Miami/1/1963	H3N8	whole virus	1.4×10^3	0.5x
A/duck/Chabarovsk/1610/1972	H3N8	whole virus	1.6×10^3	0.5x
A/duck/Ukraine/1963	H3N8	whole virus	4.1×10^5	137x
A/blue-winged teal/Alberta/346/2007	H4N3	whole virus	3.4×10^3	1x
A/mallard/Alberta/35/2001	H4N6	whole virus	3.9×10^3	1x
A/shorebird/Delaware/309/2008	H4N6	whole virus	3.3×10^5	111x
A/red knot/Delaware/541/1988	H4N6	whole virus	1.3×10^5	42x
A/common magpie/Hong Kong/645/2006	H5N1	genomic RNA	3.1×10^4	11x
A/Vietnam/1194/2004	H5N1	genomic RNA	3.1×10^4	11x
A/chicken/Egypt/M7217B/2013	H5N1	genomic RNA	3.8×10^3	1x
A/chicken/Egypt/Q1089E/2010	H5N1	genomic RNA	8.6×10^3	3x
A/Cambodia/X0810301/2013	H5N1	genomic RNA	4.2×10^3	1x
A/duck/Bangladesh/19097/2013	H5N1	genomic RNA	4.4×10^3	2x
A/Egypt/N04915/2014	H5N1	genomic RNA	3.3×10^3	1x
A/Indonesia/NIHRD11771/2011	H5N1	genomic RNA	1.0×10^4	4x
A/chicken/Bangladesh/11rs1984-30/2011	H5N1	genomic RNA	3.6×10^3	1x
A/duck/Pennsylvania/10218/1984	H5N2	genomic RNA	8.4×10^3	3x
A/chicken/Vietnam/NCVD-14-A324/2014	H5N6	genomic RNA	2.4×10^4	8x
A/shorebird/Delaware/101/2004	H5N7	genomic RNA	3.6×10^5	124x
A/shorebird/Delaware Bay/230/2009	H6N1	whole virus	3.0×10^3	1x
A/ruddy turnstone/Delaware/293/2006	H6N2	whole virus	2.5×10^5	84x
A/shorebird/Delaware/124/2001	H6N2	whole virus	4.2×10^3	1x
A/mallard/Alberta/203/1992	H6N5	whole virus	7.2×10^4	24x
A/chicken/Italy/1285/2000	H7N1	genomic RNA	2.9×10^3	1x
A/mallard/Alberta/34/2001	H7N1	genomic RNA	2.0×10^5	68x
A/mallard/Netherlands/12/2000	H7N3	genomic RNA	3.3×10^5	109x
A/chicken/Jalisco/12283/2012	H7N3	genomic RNA	1.6×10^4	5x
A/laughing gull/Delaware Bay/42/2006	H7N3	genomic RNA	3.1×10^6	1024x
A/chicken/Chile/176822/2002	H7N3	genomic RNA	4.1×10^5	133x
A/ruddy turnstone/Delaware Bay/290/2006	H7N4	genomic RNA	6.1×10^3	2x
A/mallard/Alberta/26/2001	H7, N1, N3, N5*	genomic RNA	3.9×10^3	1x
A/Netherlands/33/2003	H7N7	genomic RNA	3.6×10^5	124x
A/seal/Massachusetts/1/1980	H7N7	genomic RNA	6.9×10^3	2x
A/Shanghai/1/2013	H7N9	genomic RNA	4.8×10^4	16x
A/mallard/Alberta/194/1992	H8N4	whole virus	5.2×10^3	2x
A/mallard duck/Alberta/743/1983	H9N1	whole virus	3.0×10^3	1x
A/shorebird/Delaware Bay/133/2002	H9N1	whole virus	1.4×10^5	49x
A/quail/Hong Kong/G1/1997	H9N2	whole virus	2.1×10^3	1x
A/quail/Lebanon/272/2010	H9N2	genomic RNA	4.7×10^3	2x
A/chicken/Beijing/1/1994	H9N2	genomic RNA	2.2×10^4	8x
A/Hong Kong/33982/2009	H9N2	genomic RNA	5.1×10^4	17x
A/chukar/Shantou/22116/2005	H9N2	genomic RNA	2.1×10^4	7x
A/chicken/Hong Kong/NT10/2011	H9N2	genomic RNA	5.5×10^4	18x
A/duck/Hong Kong/Y280/1997	H9N2	whole virus	2.3×10^5	79x
A/Hong Kong/308/2014	H9N2	genomic RNA	7.0×10^6	2320x
A/shorebird/Delaware Bay/246/2003	H9N5	whole virus	6.0×10^3	2x
A/ruddy turnstone/Delaware/510/1988	H9N6	whole virus	3.3×10^5	111x

(*continued on next page*)

Table 2 (*continued*)

Strain	Subtype	Material Input into Extraction	Concentration (approx. genome copies/mL)	Multiple of LOD tested
A/gray plover/Chile/C1313/2015	H9N7	whole virus	4.4×10^5	147x
A/shorebird/Delaware Bay/31/1996	H9N7	whole virus	3.9×10^3	1x
A/shorebird/Delaware Bay/277/2000	H9N7	whole virus	4.2×10^3	1x
A/ruddy turnstone/Virginia/2297/1988	H9N9	whole virus	3.4×10^3	1x
A/mallard/Wisconsin/4230/2009	H10N1	whole virus	2.9×10^5	97x
A/shorebird/Delaware Bay/338/2009	H10N1	whole virus	1.5×10^4	5x
A/shorebird/Delaware Bay/63/1996	H10N2	whole virus	3.2×10^3	1x
A/shorebird/Delaware/260/2000	H10N4	whole virus	1.2×10^5	39x
A/chicken/Germany/N/1949	H10N7	whole virus	3.0×10^3	1x
A/mallard/Illinois/10OS4334/2010	H10N7	whole virus	1.5×10^4	5x
A/quail/Italy/1117/1965	H10N8	whole virus	5.1×10^4	17x
A/shorebird/Delaware Bay/216/1999	H11N2	whole virus	2.5×10^5	84x
A/ruddy turnstone/Delaware Bay/39/1994	H11N3	whole virus	5.8×10^5	194x
A/duck/England/1956	H11N6	whole virus	3.9×10^3	1x
A/mallard/Alberta/125/1999	H11N6	whole virus	3.0×10^3	1x
A/laughing gull/Delaware/2/2002	H11N9	whole virus	4.8×10^3	2x
A/shorebird/Delaware/6/2002	H11N9	whole virus	5.4×10^5	181x
A/American green-winged teal/Mississippi/300/2010	H11N9	whole virus	2.2×10^5	74x
A/common goldeneye/Iowa/3192/2009	H11N9	whole virus	1.4×10^5	49x
A/mallard/Ohio/1688/2009	H12N5	whole virus	3.2×10^3	1x
A/mallard/Wisconsin/4218/2009	H12N5	whole virus	7.3×10^3	3x
A/northern pintail/Missouri/319/2009	H12N5	whole virus	3.0×10^3	1x
A/northern shoveler/Mississippi/09OS025/2009	H12N5	whole virus	3.7×10^3	1x
A/ring-billed gull/Quebec/02434-1/2009	H13N6	whole virus	2.3×10^5	79x
A/black-legged kittiwake/Quebec/02838-1/2009	H13N6	whole virus	5.9×10^4	20x
A/wedge tailed shearwater/Western Australia/2327/1983	H15N9	whole virus	3.7×10^3	1x
A/shearwater/Australia/2576/1979	H15N9	whole virus	9.5×10^4	32x

* This sample is a mixed infection of H7N1, H7N3, and H7N5.

2.6. Retrospective supplemental studies

A total of 98 retrospective nasal swabs or NPS specimens known to be positive for influenza, collected from 2010 to 2018, were obtained to increase the number of influenza positive specimens for A/H1N1pdm2009 and B/Yamagata. An additional one hundred and ten (110) NPS specimens were also sourced retrospectively to increase the number of specimens collected from patients in the 65+ age bracket.

2.7. Non-seasonal influenza A performance validation

Non-seasonal influenza A was not detected in the clinical cohort, and performance was assessed using 220 blinded contrived samples. This sample set included 100 non-seasonal influenza A samples representing 41 unique influenza A subtypes. The 100 mock clinical samples were prepared by spiking each unique non-seasonal influenza A strain into a unique individual clinical negative NPS specimen purchased from Discovery Life Sciences. Fifty (50) of the 100 unique non-seasonal influenza A viruses were tested at concentrations from approximately 0.5x to 3x LOD (LOD reported in [7]), and the other 50 were analyzed at concentrations greater than 3x LOD (see Table 2 for concentration in approximate genome copies/mL). Depending on availability, some virus strains were utilized as extracted RNA and not intact whole virus. To maintain the integrity of naked RNA, clinical negative was combined with lysis buffer before the RNA was added to the mixture. The contrived samples were then extracted according to the manufacturer's instructions. To reduce potential bias, 10 contrived specimens from seasonal target virus groups and 110 influenza-negative clinical specimens were also included in the randomized, blinded sample set.

Performance of the FluChip-8G Influenza A+B Assay was assessed using a combined reference method comprised of virus characterization provided on Certificates of Analysis for commercially obtained strains, sequencing information for the HA and NA gene segments, and/or the CDC Human Flu Dx Panel of assays. A list of all non-seasonal influenza A strains and concentrations tested in this study can be found in Table 2. All influenza strains were purchased from commercial sources (Zeptometrix, International Reagent Resource, BEI Resources), or provided by the CDC or St. Jude Children's Research Hospital.

3. Results

3.1. Prospective clinical study

Of the 1065 specimens enrolled in the prospective clinical study, 43 specimens were excluded from performance analysis due to a failure to meet the inclusion criteria, resulting in a total of 1022 specimens with a final valid FluChip-8G Influenza A+B Assay result. Demographic details for the 1022 prospective specimens (700 nasal swabs and 322 NPS) and testing site specimen distribution are shown in Table 3.

Of the 1022 prospective specimens with a final valid FluChip-8G Influenza A+B Assay result, 38 specimens were excluded from analysis due to internal control failures in either the FluChip-8G Influenza A+B Assay or the comparator assay. Sensitivity and specificity for the 984 prospective specimens (664 nasal swabs and 320 NPS) are shown in Table 4. The performance of nasal swabs and NPS specimens was

Table 3
Prospective clinical study - patient demographics and testing site specimen distribution.

	Testing Site 1 N = 336 (32.9%)	Testing Site 2 N = 320 (31.3%)	Testing Site 3 N = 366 (35.8%)	Overall N = 1022
Sex				
Male	153 (45.5%)	148 (46.3%)	166 (45.4%)	467 (45.7%)
Female	183 (54.5%)	171 (53.4%)	198 (54.1%)	552 (54.0%)
Unknown	–	1 (0.3%)	2 (0.5%)	3 (0.3%)
Age (yrs)				
≤ 5 years	143 (42.5%)	146 (45.6%)	143 (39.1%)	432 (42.3%)
6 to 21 years	98 (29.2 %)	85 (26.6%)	116 (31.7%)	299 (29.2%)
22 to 59 years	84 (25.0%)	70 (21.9%)	76 (20.7%)	230 (22.5%)
≥ 60 years	11 (3.3%)	19 (5.9%)	31 (8.5%)	61 (6.0%)

Table 4
Prospective clinical study - performance of FluChip-8G Influenza A + B Assay as compared to the comparator assay.

Target Virus Group (Analyte)	Sensitivity			Specificity		
	TP/(TP + FN)	%	95% CI	TN/(TN + FP)	%	95% CI
Flu A (Overall)	51/58	87.9%	77.1 – 94.0	915/926	98.8%	97.9 – 99.3
Flu A/H1N1pdm2009	3/3	100.0%	43.9 – 100.0	975/981	99.4%	98.7 – 99.7
Flu A/H3N2	48/55	87.2%	76.0 – 93.7	924/929	99.5%	98.7 – 99.8
Flu A/Non-Seasonal	0/0	–	–	983/984*	99.9%	99.4 – 100.0
Flu B (Overall)	22/22	100.0%	85.1 – 100.0	960/962	99.8%	99.2 – 99.9
Flu B/Victoria	14/14	100.0%	78.5 – 100.0	970/970	100.0%	99.6 – 100.0
Flu B/Yamagata	8/8	100.0%	67.6 – 100.0	976/976	100.0%	99.6 – 100.0

* One (1) dual infection occurred in the course of the study, resulting in both an influenza A/H3N2 and influenza A/non-seasonal identification by the FluChip-8G Influenza A + B assay; the comparator assay resulted in Flu A/H3.

examined and no statistically significant difference in performance was observed between the two specimen types (data not shown).

One dual infection was observed in the study, resulting in both a Flu A/H3N2 result and Flu A/non-seasonal result by the FluChip-8G Influenza A + B Assay, and a result of Flu A/H3 by the comparator. Because the first FluChip-8G Influenza A + B Assay result obtained for this specimen was dual identification of A/H3N2 and A/non-seasonal, the assay was repeated in accordance with follow-up recommendations. Upon repeat, this specimen produced the same dual infection result. Reflexive bi-directional sequencing for this specimen produced a result of Flu A/H3N2, matching the result produced by the comparator assay. The FluChip-8G Influenza A + B Assay therefore correctly identified the Flu A/H3N2 virus present and the Flu A/non-seasonal result was considered a false positive. This was the only false positive result for non-seasonal A encountered in the study.

Of the 10 other specimens that were successfully sequenced, only one produced a different result than those generated by the comparator assay. The specimen was identified as influenza negative by the comparator assay, and Flu B/Victoria by sequencing. The FluChip-8G Influenza A + B Assay identified the specimen as Flu B positive, but did not identify the lineage.

3.2. Supplemental study on prospectively collected specimens

Due to the low prevalence of influenza positive specimens in the prospective clinical study, 500 additional NPS specimens were included in testing. Demographic details for the 500 supplemental clinical specimens as well as testing site specimen distribution are shown in Table 5.

Sensitivity and specificity for the 500 NPS specimens are shown in the Table 6. No invalid results were observed in this study.

Of the specimens with discordant results, sequencing identified 2 specimens that produced a different result than the comparator assay. Both specimens were identified as influenza negative by the comparator assay. Reflexive sequencing identified them as B/Yamagata and H1N1pdm2009, with the FluChip-8G Influenza A + B Assay identifying both specimens correctly.

Table 5
Supplemental clinical study - patient demographics and testing site specimen distribution.

	Testing Site 1 N = 177 (35.4%)	Testing Site 2 N = 162 (32.4%)	Testing Site 3 N = 161 (32.2%)	Overall N = 500
Sex				
Male	91 (51.4%)	79 (48.8%)	65 (40.4%)	235 (47.0%)
Female	86 (48.6%)	83 (51.2%)	96 (59.6%)	265 (53.0%)
Age (yrs)				
≤ 5 years	83 (46.9%)	67 (41.4%)	74 (46.0%)	224 (44.8%)
6 to 21 years	94 (53.1%)	94 (58.0%)	86 (53.4%)	274 (54.8%)
22 to 59 years	–	1 (0.6%)	1 (0.6%)	2 (0.4%)
≥ 60 years	–	–	–	–

3.3. Retrospective supplemental studies

A retrospective study was conducted, given the lower numbers of positive specimens for A/H1N1pdm2009 and B/Yamagata in the prospectively collected cohort. Of the 98 specimens included in retrospective testing, one was removed from the performance analysis due to an internal control failure by the FluChip-8G Influenza A + B Assay, resulting in 97 specimens included in the performance analysis (see Table 7).

Another retrospective study of 110 specimens was conducted to address the lower than expected numbers of specimens collected from patients 65 years of age or older in the prospectively collected cohort. Two specimens were removed from performance analysis due to internal control failures by either the FluChip-8G Influenza A + B Assay or comparator assay. The results for the analysis of the remaining 108 specimens are shown in Table 8.

3.4. Overall clinical study results

The combined performance observed for the FluChip-8G Influenza A + B Assay from all clinical studies conducted is shown in Table 9.

3.5. Influenza A non-seasonal performance validation

Due to the rarity of non-seasonal influenza A infections in humans, a separate validation study was executed to evaluate the performance of the non-seasonal influenza A target group by the FluChip-8G Influenza A + B assay. This testing included 100 contrived non-seasonal influenza A strains, 110 influenza negative specimens, and 10 contrived seasonal influenza positive samples. Of the 220 blinded samples analyzed, 7 influenza negative samples were removed from performance analysis due to internal control failures by either the comparator assay or FluChip-8G Influenza A + B assay. FluChip-8G Influenza A + B Assay performance for the remaining 213 contrived specimens can be found in Table 10.

One pre-2009 H1N1 strain (A/Fukushima/141/2006) analyzed at approximately 1.5x LOD produced a false positive result for A/H1N1pdm2009.

4. Discussion

Overall concordance analysis of the data from the combined clinical studies resulted in clinical performance that exceeded 99% specificity for all target virus groups for the FluChip-8G Influenza A + B Assay when compared to the CDC Human Flu Dx Panel (Table 9). The sensitivity for both influenza B lineages and influenza A/H1N1pdm2009 were greater than 95%. The H3N2 target virus group had the lowest

Table 6
Supplemental clinical study - performance of FluChip-8G Influenza A + B Assay as compared to the comparator assay.

Target Virus Group (Analyte)	Sensitivity			Specificity		
	TP/(TP + FN)	%	95% CI (LCL – UCL)	TN/(TN + FP)	%	95% CI (LCL – UCL)
Flu A (Overall)	189/204	92.6%	88.2 - 95.5	293/296	98.9%	97.1 – 99.7
Flu A/H1N1pdm2009	24/26	92.3%	75.9 – 97.9	469/474	98.9%	97.6 – 99.5
Flu A/H3N2	163/175	93.1%	88.4 – 96.0	325/325	100.0%	98.8 – 100.0
Flu A/Non-Seasonal	0/0	–	–	500/500	100.0%	99.2 – 100.0
Flu B (Overall)	52/53	98.1%	90.1 – 99.7	444/447	99.3%	98.0 – 99.8
Flu B/Victoria	46/46	100.0%	92.3 – 100.0	452/454	99.6%	98.4 – 99.9
Flu B/Yamagata	6/7	85.7%	48.7 – 97.4	492/493	99.8%	98.9 – 100.0

Table 7
Retrospective clinical study - performance of FluChip-8G Influenza A + B Assay testing retrospective A/H1N1pdm2009 and B/Yamagata specimens as compared to the comparator assay.

Target Virus Group (Analyte)	Sensitivity			Specificity		
	TP/(TP + FN)	%	95% CI (LCL – UCL)	TN/(TN + FP)	%	95% CI (LCL-UCL)
Flu A (Overall)*	24/25	96.0%	80.5 – 99.3	72/72	100.0%	94.9 – 100.0
Flu A/H1N1pdm2009	24/24	100.0%	86.2 – 100.0	73/73	100.0%	95.0 – 100.0
Flu A/H3N2	0/0	–	–	97/97	100.0%	96.2 – 100.0
Flu A/Non-Seasonal	0/0	–	–	97/97	100.0%	96.2 – 100.0
Flu B (Overall)**	63/65	96.9%	89.5 – 99.2	32/32	100.0%	89.3 – 100.0
Flu B/Victoria	0/0	–	–	96/97	99.0%	94.4 – 99.8
Flu B/Yamagata	62/64	96.8%	89.3 – 99.1	33/33	100.0%	89.6 – 100.0

* Flu A (Overall) category includes a specimen that resulted in a comparator assay result of 'Flu B/ Yamagata and Flu A/ No Subtyping'. This specimen was identified as 'Flu B/Yamagata' by the FluChip-8G Influenza A + B Assay.

** Flu B (Overall) category includes a specimen that resulted in a comparator assay result of 'Flu B (no lineage).' This specimen was identified as 'Flu B/Victoria' by the FluChip-8G Influenza A + B Assay.

sensitivity of 91.8%. Most of the specimens for which a false negative was obtained by the FluChip-8G Influenza A + B Assay are low concentration specimens, as evidenced by high associated CDC Human Flu Dx Panel Flu A and Flu B Ct values (data not shown). Given that the CDC assay is run as a series of singleplex assays whereas the FluChip-8G Influenza A + B Assay is a multiplexed assay, it is not surprising that the FluChip-8G Influenza A + B Assay exhibits slightly lower clinical sensitivity than the CDC Human Flu Dx Panel of assays.

One specimen in the clinical studies resulted in a dual infection by the FluChip-8G Influenza A + B Assay, but only a single infection by the CDC Flu Dx Assay (corroborated by reflexive bi-directional sequencing). There is a possibility that if two viruses were present, with one at significantly lower concentration, the undetected virus may have been below the detection limit of the sequencing method used. While it is still technically possible that this specimen may have been a dual infection, the probability of this is low. Only 1 specimen out of all 1689 specimens tested produced a false positive result of influenza A/non-seasonal, indicating high specificity for the influenza A non-seasonal target virus group. Nevertheless, there is known genetic overlap between seasonally-circulating influenza A viruses and emerging viruses, such as H1N1v and H3N2v [8,9]. Therefore, a single infection of variant viruses (H1N1v and H3N2v) may generate a dual positive result of both seasonal and "non-seasonal" influenza A by the FluChip-8G Influenza A + B Assay [7].

While the true clinical sensitivity for the A/non-seasonal category was unable to be assessed in the clinical study due to the absence of non-seasonal influenza A virus infections in the clinical study cohort, a non-seasonal influenza A validation study was executed to assess performance. There was one misidentified influenza A non-seasonal strain in this study. A likely cause for the false positive observed in this specimen was that the concentration of human cellular DNA was 2 orders of magnitude higher than the concentration of the influenza A RNA, indicating competition between the internal control and influenza amplification. This study correctly identified the other 99 out of 100 unique, blinded influenza A non-seasonal strains, including 49 diverse strains that were correctly identified when analyzed near the LOD, demonstrating wide-ranging inclusivity of the assay for this target virus group.

Although there were no observed non-seasonal viruses in the clinical study cohort, the emergence of novel influenza viruses occurs

Table 8
Retrospective clinical study - performance of FluChip-8G Influenza A + B Assay testing retrospective specimens from patients aged 65 + as compared to the comparator assay.

Target Virus Group (Analyte)	Sensitivity			Specificity		
	TP/(TP + FN)	%	95% CI (LCL – UCL)	TN/(TN + FP)	%	95% CI (LCL-UCL)
Flu A (Overall)	17/18	94.4%	74.2 – 99.0	90/90	100.0%	95.9 – 100.0
Flu A/H1N1pdm2009	3/3	100.0%	43.9 – 100.0	105/105	100.0%	96.5 – 100.0
Flu A/H3N2	14/15	93.3%	70.2 – 98.8	93/93	100.0%	96.0 – 100.0
Flu A/Non-Seasonal	0/0	–	–	108/108	100.0%	96.6 – 100.0
Flu B (Overall)	11/12	91.6%	64.6 – 98.5	96/96	100.0%	96.2 – 100.0
Flu B/Victoria	0/0	–	–	108/108	100.0%	96.6 – 100.0
Flu B/Yamagata	11/12	91.6%	64.6 – 98.5	96/96	100.0%	96.2 – 100.0

Table 9
Overall clinical study results - performance of FluChip-8G Influenza A + B Assay as compared to the comparator assay (N = 1689).

Target Virus Group (Analyte)	Sensitivity			Specificity		
	TP/(TP + FN)	%	95% CI (LCL – UCL)	TN/(TN + FP)	%	95% CI (LCL – UCL)
Flu A (Overall)*	281/305	92.1%	88.6 – 94.7	1370/1384	99.0%	98.3 – 99.4
Flu A/H1N1pdm2009	54/56	96.4%	87.9 – 99.0	1622/1633	99.3%	98.8 – 99.6
Flu A/H3N2	225/245	91.8%	87.7 – 94.7	1439/1444	99.7%	99.2 – 99.9
Flu A/Non-Seasonal	0/0	–	–	1688/1689**	99.9%	99.7 – 100.0
Flu B (Overall)***	148/152	97.4%	93.4 – 99.0	1532/1537	99.7%	99.2 – 99.9
Flu B/Victoria	60/60	100.0%	94.0 – 100.0	1626/1629	99.8%	99.5 – 99.9
Flu B/Yamagata	87/91	95.6%	89.2 – 98.3	1597/1598	99.9%	99.7 – 100.0

* Flu A (Overall) category includes a specimen that resulted in a comparator assay result of 'Flu B/Yamagata and Flu A/No Subtyping'.

** One (1) dual infection occurred during the study, resulting in both an influenza A/H3N2 and influenza A/Non-Seasonal identification by the FC8G assay, a result of only Flu A/H3N2 by the comparator method.

*** Flu B (Overall) category includes a specimen that resulted in a comparator assay result of 'Flu B (no lineage).' This specimen was identified as 'Flu B/Victoria' by the FluChip-8G Influenza A + B Assay.

Table 10
Non-seasonal influenza A challenge study overall performance.

Target Virus Group (Analyte)	Sensitivity			Specificity		
	TP/ (TP + FN)	%	95% CI(LCL – UCL)	TN/ (TN + FP)	%	95% CI (LCL – UCL)
Flu A (Overall)	105/105	100.0%	96.5 – 100.0	107/108	99.1%	94.9 – 99.8
Flu A/H1N1pdm2009	2/2	100.0%	34.2 – 100.0	209/211	99.1%	96.6 – 99.7
Flu A/H3N2	3/3	100.0%	43.9 – 100.0	210/210	100.0%	98.2 – 100.0
Flu A/Non-Seasonal	**99/100**	**99.0%**	**94.6 – 99.8**	**113/113**	**100.0%**	**96.7 – 100.0**
Flu B (Overall)	5/5	100.0%	56.6 – 100.0	208/208	100.0%	98.2 – 100.0
Flu B/Victoria	2/2	100.0%	34.2 – 100.0	211/211	100.0%	98.2 – 100.0
Flu B/Yamagata	3/3	100.0%	43.9 – 100.0	210/210	100.0%	98.2 – 100.0

intermittently in humans, including during the 2009 pandemic and at county and state fairs where variant viruses have been transmitted from pigs to humans [10,11]. There is always a risk of an emergent pandemic virus and early identification of non-seasonal influenza outbreaks is critical to both regional and global public health. The FluChip-8G Influenza A + B Assay provides identification of seasonal viruses in the same assay as viruses with pandemic potential without additional reflexive testing and has demonstrated detectivity on a broad range of non-seasonal influenza viruses. In addition, it is expected that the FluChip-8G Influenza A + B Assay will remain robust as new variant viruses emerge due, in-part, to the highly conserved regions in which the primers were designed. The primers for many existing molecular diagnostics target very specific regions of the influenza genome that either can mutate or are not present in other subtypes. In the event a new variant virus emerges, these assays have a higher risk of failing to detect the new influenza virus due to a failure in priming [2]. The primers in the FluChip-8G Influenza A + B Assay target conserved regions of influenza genome, and amplify entire gene segments regardless of subtype or lineage, allowing for detection and discrimination of a wide variety of seasonal and non-seasonal viruses. Because of this universal amplification, the discrimination of subtype and/or lineage arises from the microarray hybridization and not during the amplification step. Therefore, if a novel virus emerges that the assay does not recognize, it would require little effort to modify the FluChip-8G software to detect the new influenza pattern and to specifically identify an emerging virus without changing the underlying assay. While the FluChip-8G Influenza A + B Assay is already positioned for the next pandemic, this method of interpretation enables the assay to be rapidly updated, tested, and deployed if necessary.

This assay has utility in a clinical or public health laboratory as the operators in these settings are familiar with nucleic acid extraction and RT-PCR amplification, and can be easily trained in microarray processing. Up to 44 specimens can be analyzed by a single operator within 9 h, with 3.5–5 hours of hands-on time, depending on the number of specimens being processed. Cross-contamination of nucleic acid can occur if proper techniques and workflow for the execution of molecular assays are not employed. However, contamination is minimal for this assay when proper procedures and workflows for executing molecular assays are implemented (data not shown) [12].

A single multiplexed assay capable of detecting both seasonal influenza viruses as well as potential emerging viruses of zoonotic origin with good sensitivity and specificity with a same-day turnaround time has important utility both clinically and in public health. We hope the availability of the FluChip-8G Influenza A + B Assay may enable more timely detection of potential emerging viruses from human clinical specimens to increase pandemic preparedness while also detecting more typical clinically-relevant influenza virus infections.

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Declaration of Competing Interest

RHB, EDD, AWT, JEJ, AHS, KO, AWS, ET, KL, RSS, and KLR are current or former employees of InDevR. EDD, AWS, and KLR are stockholders of InDevR, Inc. AWT, EDD, AWS, RHB, RSS, and KLR are named inventors on patent applications related to the material herein.

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