Evaluation of Alere i Influenza A&B for Rapid Detection of Influenza Viruses A and B

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**Running head:** Alere i Influenza A&B Rapidly Detects Influenza Viruses

This study was presented in part at the Association for Molecular Pathology 2013 Annual Meeting, Phoenix, AZ, 14-16 November, 2013

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Abstract

Rapid and accurate diagnosis of influenza (Flu) is important for infection control as well as for patient management. Alere i Influenza A&B is an isothermal nucleic acid amplification-based integrated system for detection and differentiation of FluA and FluB. The performance of the Alere i Influenza A&B was screened using frozen nasopharyngeal swab specimens collected in viral transport media (VTM) that were originally tested fresh with the FilmArray Respiratory Panel (RP) assay during the 2012/2013 influenza outbreak. In total, 360 VTM specimens were selected for Alere i Influenza A&B testing, including 40 FluA H1N1-2009 (FluA-1), 40 FluA H3N2 (FluA-3), 37 FluA “equivocal” or “no subtype detected” (FluA-u), 41 FluB and 202 Flu-negatives as initially determined by the FilmArray RP assay. The Alere assay showed sensitivities of 87.2%, 92.5%, 25.0% and 97.4% for FluA-1, FluA-3, FluA-u, and FluB, respectively, after discordant resolution by Prodesse ProFLU+ PCR. The specificities were 100% for both FluA and FluB. In general, the Alere i Influenza A&B provided good sensitivity although the assay did show poorer sensitivity with samples determined to be of low FluA titer by Prodesse ProFLU+ PCR (mean real-time PCR threshold cycle (C\(_T\)) value of 31.9 ± 2.0), which included the majority of the samples called FluA “equivocal” or “no subtype detected” by a single BioFire FilmArray RP test. The integrated, rapid and simple characteristics of the Alere i Influenza A&B make it potential for point-of-care testing with a test turnaround time less than 15 minutes.

Keywords: Influenza viruses A and B, nicking endonuclease amplification reaction, point-of-care testing
**Introduction**

Influenza (Flu) is a contagious respiratory illness caused by influenza viruses A and B in humans. The clinical presentation of the disease ranges from asymptomatic infection to severe complications including viral pneumonia and death, especially in immunocompromised patients, patients with underlying comorbidities, and those at the extremes of age (1-3). Since the 2009 pandemic, Flu A 2009 H1N1 (FluA-1) has quickly become a dominant influenza strain circulating throughout the world along with seasonal FluA H3N2 (FluA-3) and FluB (4, 5).

Typical symptoms of influenza include fever, cough, sore throat, rhinorrea and nasal congestion - symptoms which overlap with other viruses circulating at the same time (6, 7). Unlike other viruses, rapid and accurate diagnosis of influenza is necessary for prompt administration of antiviral therapy, mainly oseltamivir, which should be administered within 48 hours of symptoms onset (8). Additional benefits of rapid identification include infection control and public health notification and tracking, stopping unnecessary use of antibiotics, hospital procedures and laboratory tests (9-11).

Current diagnostic techniques for the detection and identification of Flu include rapid influenza antigen detection tests (RIDTs), direct fluorescent-antibody assays (DFAs), viral culture and nucleic acid amplification tests (NAAT). Commercially available RIDTs are widely used in clinical practice as point of care tests because they are simple to use and provide results within 15-30 minutes (12, 13). However, their sensitivities vary widely depending on the manufacturer and can be as low as 10% with specificity ranging from 90-100% (14). DFAs are more sensitive than RIDTs and can be accomplished within 3 hours but require skilled technologists for correct
Similarly, culture has increased sensitivity over both RIDTs and DFAs but requires skilled technologists, specialized laboratory settings and has a slow turn-around time (2-14 days) (17). NAAT are highly sensitive and are gradually replacing culture as the gold standard, but these tests are generally expensive and, depending on the manufacturer, require highly skilled molecular technologists with turn-around time of up to 24 hours from receipt to results (1, 7, 18-22). As a result, specimens with negative RIDTs are usually tested subsequently by more sensitive culture or molecular assays. PCR-based molecular assays have shown excellent clinical utility for the detection and identification of influenza viruses; numerous FDA-cleared commercial devices are now available (18, 23, 24).

The ideal diagnostic test for influenza would have the fast turn-around time and simplicity of an RIDT with the sensitivity and specificity of a NAAT. The Alere i Influenza A&B (Alere Scarborough, Scarborough, ME) assay incorporates a nicking endonuclease amplification reaction (NEAR) technique for the detection and differentiation of FluA and FluB in nasopharyngeal swab (NPS) specimens. The system requires only 2 minutes of total hands-on time to process and setup one sample, and results are available within 15 minutes. The objective of this study was to evaluate the clinical performance of the Alere i Influenza A&B assay on NPS specimens soaked in viral transport media (VTM) that were previously tested by the FilmArray Respiratory Panel (RP, BioFire Diagnostics, Salt Lake City, UT).
MATERIALS AND METHODS

Clinical specimens. A retrospective study was conducted on banked NPS specimens collected from inpatients presenting with flu-like symptoms at Memorial Sloan-Kettering Cancer Center (MSKCC) between December 15, 2012 and March 1, 2013 during an influenza outbreak. These NPS specimens were soaked in 3 ml of VTM and leftovers were stored at -80°C after the single FilmArray RP assay was performed. Based on the FilmArray results, up to 40 specimens positive for each virus and genotype including FluA-1, FluA-3, FluA “equivocal” or “untypeable” (FluA-u) and FluB were selected. For each Flu-positive specimen, 1-2 upstream and/or downstream Flu-negative specimens based on their accession numbers were also selected. Duplicate specimens from the same patient were later excluded. The study was approved by the MSKCC Institutional Review Board with waiver of HIPAA authorization and informed consent.

Alere i Influenza System. The Alere i Influenza System deploys the NEAR technique to detect and distinguish FluA and FluB. NEAR incorporates an isothermal nucleic acid amplification technology to provide ultra-rapid DNA or RNA amplification, with results in ten minutes or less when coupled to fluorescence-based detection (US Patents: US2009/0017453, US2009/0081670). Two templates (primers) and three enzymes (a thermostable DNA polymerase, a reverse transcriptase, and a thermostable nicking endonuclease) provide the driving force behind NEAR when the target is RNA. In conjunction with the two templates, these enzymes provide a method for exponential amplification of short amplicons that combines satisfactory specificity with a very rapid time frame. Detection is accomplished in real time, using fluorescently-labeled molecular beacons (Figure 1).
The Alere System was performed as instructed by the manufacturer. Briefly, the Sample Receiver and Test Base were inserted into the instrument and the lysis buffer in the Sample Receiver was automatically heated by the instrument (Figure 2). After approximately three minutes, an aliquot of sample from the VTM (0.2 ml) was transferred into the Sample Receiver and mixed by pipetting. Two 0.1 ml aliquots of the eluate from the Sample Receiver were then manually transferred via the Transfer Cartridge to the Test Base to rehydrate the lyophilized NEAR InfA and InfB reaction mixes and initiate target amplification and detection. Heating, agitation and detection by fluorescence is performed automatically by the instrument. The control results are automatically checked by the reader to ensure that the test result is valid. Results are reported for influenza A and B viruses as negative or positive within 10-12 minutes. As required by the study protocol, a positive control and a negative control swabs provided by the manufacturer were run each day before patient specimens were tested.

**FilmArray RP assay.** The specimens were transported within 2 hours to the microbiology laboratory, where they were processed immediately for routine respiratory pathogen diagnosis. The FilmArray RP (version 1.6) was performed according to the manufacturer’s instructions and as previously described (19). Briefly, 1 ml of hydration solution was injected into the FilmArray pouch to rehydrate the reagents. Using a transfer pipette, approximately 300 µl of respiratory sample was added to the sample buffer vial, and the resulting mixture was transferred to the pouch using the sample loading syringe. The pouch was then placed in the FilmArray instrument, and a preprogrammed PCR run was initiated.
Prodesse ProFLU+. The ProFLU+ assay (GenProbe/Hologic H44VK00) was performed on a subset of enrolled NPS/VTM specimens according to the manufacturer’s instructions, with the following modifications (25, 26). Sample processing was performed on the Qiacube work station (Qiagen, Valencia, CA) using the QiaAmp Viral RNA Mini kit. PCR was performed on the Roche LightCycler 480 (Roche Diagnostics, Indianapolis, IN). The data analysis and report were blinded to FilmArray and Alere results.

Evaluation standards and data analysis. Specimens were considered positive or negative when results from the Alere assay matched the FilmArray RP. Discrepant results were resolved based on the Prodesse ProFLU+ results. McNemar’s test was used to compare the Alere and the reference results and Student t test was used to compare cycle threshold (Ct) value means. Statistical analysis was calculated from www.graphpad.com/quickcalcs. A P value of ≤0.05 was considered statistically significant.
RESULTS

During the study period, 3,675 NPS specimens were submitted and tested by a single FilmArray RP assay. Among them, 45, 425, 37 and 77 were tested positive for FluA-1, FluA-3, FluA-u, and FluB, respectively. Based on the selection criteria, a total of 360 specimens were enrolled in this study. These specimens were collected from 193 female and 167 male patients with 69 (19.2%) children (<18 years old) and 291 (80.8%) adults. They included 40 FluA-1, 40 FluA-3, 37 FluA-u (21 “equivocal”, 16 “untyperable”), 41 FluB and 202 Flu-negative as determined by the FilmArray RP. Among the 202 Flu-negative samples tested by FilmArray, 60 were positive for one or more non-Flu viruses including 21 rhinoviruses/enteroviruses, 13 respiratory syncytial viruses and 14 coronaviruses (229E, 5; OC43, 4; NL63, 4; HKU1, 1).

Among the 360 enrolled specimens, the single Alere assay reported 79 FluA, 37 FluB, 240 negatives and four invalid results (failure of internal controls) (Table 1). A single ProFLU+ was performed on all 42 discordant specimens and 37 concordant specimens (based on Alere and FilmArray). Results from all 37 concordant samples were confirmed by ProFLU+. Among the 42 discordants, ProFLU+ detected 31/41 (8 FluA, 21 FluA-u and 2 Flu B) which were positive by the FilmArray RP, and 1/1 positive (FluA) by the Alere.

Using a combination standard defined as concordant results of two or more of the Alere, FilmArray and ProFLU+ assays, clinical performance of the Alere assay was determined (Table 1). The performance of the Alere and reference assays for FluA-1, FluA-3 and FluB were not statistically different except for FluA-u. The specificity for FluA was 100% (95% CI, 98.5-100).
The sensitivities for the three FluA genotypes were 87.2% for FluA-1, 92.5% for FluA-3 and 25% for FluA-u. For FluB testing, the Alere assay showed excellent agreement with reference results. Only one of 38 FluB positive specimens was missed by the Alere assay. The sensitivity and specificity for FluB were 97.4% (95% CI, 86.5-99.5) and 100% (95% CI, 98.9-100), respectively.

The greatest comparative difference in sensitivity was observed for FluA-u; only 7 of 28 (25%) of these specimens were positive by the Alere assay. Discordant results were revealed more frequently for specimens with higher C\textsubscript{T} values in the FilmArray and ProFLU+ assays (Table 2). Among the 29 FluA reference-positive, Alere-negative specimens, 22 (75.9%) had C\textsubscript{T} values of 25-30 by FilmArray RP and 26 (89.7%) had C\textsubscript{T} values of 30-40 by the ProFLU+ assay. The mean C\textsubscript{T} values in FilmArray RP (27.0±2.6) and ProFLU+ (31.9 ±2.0) in specimens with discordant results were significantly higher than those obtained for concordant results (17.1±5.4 for FilmArray RP, \(P<0.01\); 30.1±1.9 for ProFLU+, \(P=0.018\)).
DISCUSSION

The Alere i Influenza A&B assay described in this study is a novel, isothermal amplification-based integrated system for detection and differentiation of FluA and FluB. It is a fast and user-friendly procedure with specimen collection to result in less than 15 minutes including two minutes hands-on time. In this study, we evaluated the performance of the Alere i assay on a panel of archived, frozen NPS VTM samples. A perfect specificity was observed for both FluA and FluB detection, which provides clinicians with the confidence to act appropriately when a positive result is obtained. FluA sensitivity observed here would translate to 87% after adjusting for the incidence of each subtype in the overall collection (N=3,675). The lowest sensitivity was observed for the small subset of specimens that were “equivocal” or “untypeable” by the FilmArray RP test; except for these, the overall FluA sensitivity would have been 92%.

Reference-positive, Alere-negative discordant results were observed more frequently for specimens with higher C_T values (i.e., lower viral loads) of FilmArray assays, suggesting that the lower detection rate by the Alere assay in FilmArray FluA-u specimens was associated with the lower viral titer in the diluted NPS VTM. Furthermore, in our study, the 291 (80.8%) NPS specimens tested were from adults who probably yielded lower viral shedding than children for many detection methods (7, 27). The Alere assay possessed an excellent performance on FluB detection; only one out of 38 FluB positive specimens was not detected by Alere i Influenza A & B, giving a high sensitivity of 97.4%.

This study has a couple of important limitations. First, the Alere assay accommodates crude swab specimens eluted directly, rather than swabs first diluted in VTM, which may reduce
detection of very low titer VTM samples. Second, the enrolled specimens had been stored frozen 6-9 months before the Alere and Prodesse testing (a circumstance not permitted in the FilmArray package insert), perhaps further reducing the already low titer specimens, and expanding the impact of sampling statistics on the results distribution. It should be noted, however, that the samples called FluA-u in this study included Biofire “equivocals” and “no subtype detected”, both of which require at least one Biofire retest before declaring the Biofire FluA status according to the FilmArray RP Instruction Booklet (i.e., Flu status ambiguous), and since this retest was not performed, the Biofire reference assay status was uncertain. Taken together, the results reported here suggest that the performance of the Alere i against the Film Array RP may depend upon sample handling and the frequency of FilmArray FluA equivocal/untypables.

The Alere i Influenza A&B assay evaluated in this study has the potential to serve as an alternative to RIDTs, with significantly improved sensitivities. The Alere i system has the advantage of a significantly shorter test time than any currently available molecular assay. The simple, pipette-free procedure runs on a fully integrated, closed small footprint system making the Alere assay potentially suitable for point-of-care testing.
Acknowledgements

We thank the Clinical Microbiology Service staff of the Memorial Sloan-Kettering Cancer Center for helping collecting clinical specimens. Rich Roth is an employee of Ionian Technologies, a subsidiary of Alere Scarborough, the commercial manufacturer of the Alere i Influenza A&B system. Prodesse PCR was performed by Brion Mermer and Nancy Turcotte, at Alere Scarborough. This study was supported in part by a research agreement between MSKCC and Alere Scarborough (SK2013-0262).
References


Figure 1. NEAR mechanism. A. Mechanism of the NEAR amplification duplex formation. (1) The recognition region of T2 binds to the complementary target region and is extended by polymerase along the target, (2) A second T2 binds to the same target and is extended, displacing the first T2 (3), (4) The recognition region of T1 binds to its complement in the released strand and is extended to the 5' end, creating a double stranded nicking enzyme recognition site, (5) Nicking enzyme binds and nicks (denoted by scissors), (6) polymerase synthesizes off the cleaved 3' OH along T1, displacing the remaining target complement, and (6) the final extended double-stranded complex is termed the NEAR amplification duplex. B. Mechanism of product formation. (1b) Nicking enzymes bind to both nicking enzyme recognition sites on the NEAR duplex; cleavage and strand displacement amplification at both sites creates two complexes, each consisting of a duplex stability region and nicking enzyme recognition region and a single-stranded target (2b), (2b + 3b) Repeated nicking, polymerization and strand displacement results in the amplification of Product 1 and 2. Cleaved complexes are regenerated (3b), while Products 1 and 2 can anneal to T1 and T2 respectively (4b) resulting in bidirectional extension creating duplexes that generate the opposite Product upon cleavage. The products continue to recycle until the templates, dNTPs, or enzymes are depleted.

Figure 2. Flowchart of the Alere i Influenza A&B assay. The total elapsed time was within 15 minutes and the total hands-on time was within 2 minutes.
Table 1. Performance of the Alere i Influenza A&B in VTM specimens compared to reference results

<table>
<thead>
<tr>
<th>Virus</th>
<th>Genotype</th>
<th>No detected: Alere / reference</th>
<th>% Sensitivity (95% CI)</th>
<th>% Specificity (95% CI)</th>
<th>Discordance</th>
<th>P Value</th>
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<td>FluA</td>
<td>+/+</td>
<td>79</td>
<td>73.2</td>
<td>100</td>
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<tr>
<td></td>
<td>+/-</td>
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<td>(64.1, 80.6)</td>
<td>(98.47, 100)</td>
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<td></td>
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<tr>
<td></td>
<td>-/+</td>
<td>29</td>
<td>87.2</td>
<td>100</td>
<td>0.073</td>
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<tr>
<td></td>
<td>--</td>
<td>248</td>
<td>(73.29, 94.4)</td>
<td>(98.8, 100)</td>
<td>366</td>
<td></td>
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<tr>
<td>H1N1-2009</td>
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<td>100</td>
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<td>(80.14, 97.42)</td>
<td>(98.8, 100)</td>
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<tr>
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<td>-/+</td>
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<td>92.5</td>
<td>100</td>
<td>&lt;0.001</td>
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<tr>
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<td>25</td>
<td>(12.68, 43.36)</td>
<td>(98.84, 100)</td>
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<tr>
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<td>(98.88, 100)</td>
<td>371</td>
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<tr>
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<td>-/+</td>
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<td>--</td>
<td>1</td>
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<td>FluB</td>
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<td>100</td>
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<tr>
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<tr>
<td></td>
<td>-/+</td>
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</table>

* Four Alere invalid results were excluded from the statistical analysis, leaving a total of 356.

* One FilmArray-negative sample identified as FluA by the Alere and confirmed by ProFLU+.
Table 2. Positivity of Alere FluA results and C<sub>T</sub> values in FilmArray RP and Pro FLU+

<table>
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<tr>
<th>Alere results</th>
<th>FilmArray RP C&lt;sub&gt;T&lt;/sub&gt; values&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mean±SD</th>
<th>Pro FLU+ C&lt;sub&gt;T&lt;/sub&gt; values&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mean±SD</th>
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<tr>
<td></td>
<td>&lt;15.0 15.0-19.9 20.0-24.9 25.0-30.0</td>
<td></td>
<td>&lt;25.0 25.0-29.9 30.0-40.0</td>
<td></td>
</tr>
<tr>
<td>Positive&lt;sup&gt;c&lt;/sup&gt;</td>
<td>30 23 15 10</td>
<td>17.1±5.4</td>
<td>1 6 4</td>
<td>29.5±2.8</td>
</tr>
<tr>
<td>Negative</td>
<td>0 0 7 22</td>
<td>27.0±2.6</td>
<td>0 6 23</td>
<td>32.0±2.0</td>
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</table>

<sup>a</sup> C<sub>T</sub> value of 30 and 40 was used to represent FilmArray RP and ProFLU+ negative result, respectively.

<sup>c</sup> One FluA positive sample determined by Alere and ProFLU+ was excluded.