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# Evaluation of Simplexa Flu A/B & RSV for Direct Detection of Influenza Viruses (A and B) and Respiratory Syncytial Virus in Patient Clinical Samples

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**We evaluated the performance of the Simplexa Flu A/B & RSV kit on 170 prospective respiratory samples using a modified protocol, supplied by the manufacturer, that eliminates the RNA extraction step. Overall, compared against our laboratory-developed assay, the assay's sensitivity, specificity, and positive and negative predictive values were 95.1%, 99.6%, 98.7%, and 98.6%, respectively.**

Respiratory tract infections (RTIs) account for a large proportion of morbidity in both pediatric and adult populations worldwide. Of the human pathogens, respiratory viruses have been shown to be the etiological agents of greater than 70% of RTIs (1). Of interest are the influenza viruses (A and B) and respiratory syncytial virus (RSV), which have been well documented to account for 40% of RTIs (2). Rapid detection of influenza viruses (A and B) and RSV is of utmost importance for patient management and for the prevention of health care-associated viral infections. Viral antigen detection assays have been shown to have a wide range of sensitivities and specificities for the detection of influenza viruses and RSV (1, 3). With the advent of nucleic acid testing and, in particular, the utilization of quantitative real-time reverse transcriptase PCR (qRT-PCR), several assays were developed for the rapid detection of RSV and influenza viruses A and B (4–7). However, one of the major drawbacks of these assays is the need for viral nucleic acid extraction from patient samples.

In the study described in this report, we evaluated the Simplexa Flu A/B & RSV kit (catalogue number MOL2600; Focus Diagnostics Inc., Cypress, CA), intended for use on the 3M integrated cycler instrument. The 3M integrated cycler is a thermocycler that is capable of heating, cooling, mixing of sample and reagents, and real-time fluorescence detection of up to four distinct analytes. Clinical samples are pipetted into appropriate sample wells on a universal disc (96 wells). The instrument utilizes disc media to contain and to process samples and uses real-time fluorometric detection to identify targets within the sample reaction wells. The disc can process up to 96 independent samples during one run.

A protocol modified by the manufacturer, Focus Diagnostics Inc. (Cypress, CA), was used for the rapid detection of influenza virus (A and B) and RSV RNA directly from patient samples. The modified protocol eliminated the need for viral RNA extraction. This helps shorten the turnaround time required to obtain the test result, reduces the chance of contaminating the sample during the extraction step, and reduces the cost of running the test. Briefly, 50-μl aliquots of patient samples were placed into either a 96-well PCR sterile plate or into flat-bottomed 2-ml Eppendorf tubes and heated in a thermocycler machine or in a heat block, respectively. It was extremely important to use flat-bottomed 2-ml tubes to ensure uniform heating of the sample and to optimize the assay's performance. Patient samples were heat treated at 70°C for exactly 5 min. Heated samples where then loaded onto the universal disc or stored at −70°C pending further analysis.

The performance of the Simplexa FluA/B & RSV kit was compared against that of our laboratory-developed assay (LDA) for the detection of influenza viruses [A, B, A (H1N1) pandemic 2009 (pdm09)] and RSV. The primers and probes utilized in our LDA were previously reported (8–11). The LDA's performance was continuously monitored by participating in the Quality Control for Molecular Diagnostics (QCMD) External Quality Assessment/Proficiency Testing programs (Scotland, United Kingdom) and the World Health Organization (WHO) External Quality Assessment Programme for the Detection of Influenza Virus A by PCR. In addition, the presence of inhibitors and the quality of the patient samples collected were investigated by determining the presence of the RNase P gene in all patient samples tested.

One hundred seventy consecutive respiratory samples (nasopharyngeal and throat swabs) collected in Σ-Virocult (Medical Wire & Equipment Co, Wiltshire, United Kingdom) liquid viral transport system from patients between 17 February 2012 and 28 February 2012 were tested by both methods. This study was part of the government-approved surveillance program for respiratory viruses that was performed at Israel National Influenza Center with Sheba Medical Center Helsinki Number SMC-9156-11. Testing the patient samples by the LDA for influenza viruses A, B, and A (H1N1) pdm09 and RSV was performed after total nucleic acid was extracted from 500 μl eluted patient sample using a NucliSENS easyMAG (bioMérieux, Marcy l'Etoile, France) semiautomated extractor and eluted in 55 μl elution buffer. The LDA 25-μl total reaction volume was prepared by mixing 20 μl AgPath-ID one-step RT-PCR reagents (Life Technology) containing the assay primers and probes [influenza A virus primers and probes, 300 nM and 200 nM final concentrations, respectively

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**TABLE 1** Comparison between LDA and Focus Simplexa FluA/B and RSV kit[a]

| Target virus | No. of viruses with the following result by LDA/Simplexa kit: | | | | Positive agreement | | Negative agreement | |
|---|---|---|---|---|---|---|---|---|
| | +/+ | +/− | −/+ | −/− | Sens. (%) | PPV (%) | Spec. (%) | NPV (%) |
| Influenza A virus | 28 | 2 | 0 | 92 | 93.3 | 100.0 | 100.0 | 97.9 |
| Influenza B virus | 30 | 0 | 1 | 92 | 100.0 | 96.8 | 98.9 | 100.0 |
| RSV | 19 | 2 | 0 | 92 | 90.5 | 100.0 | 100.0 | 97.9 |
| Overall | 77 | 4 | 1 | 276 | 95.1 | 98.7 | 99.6 | 98.6 |

[a] Sens., sensitivity; Spec., specificity; PPV, positive predictive value; NPV, negative predictive value.

(10); influenza B virus primers and probe, 300 nM and 200 nM final concentrations, respectively (11); A (H1N1) pdm09 primers and probe, 400 nM and 200 nM final concentrations, respectively (8); RSV group A (RSV-A) primers and probe, 180 nM and 50 nM final concentrations, respectively, and RSV-B primers and probe, 300 nM and 50 nM final concentrations, respectively (9)] and 5 μl of the RNA extracted from the patient. The sensitivity and specificity of the LDA qRT-PCR multiplex assay compared to the results of singleplex qRT-PCR assays were both 100%.

In every real-time PCR run, positive [influenza viruses A and B, A (H1N1) pdm09, RSV-A, and RSV-B] and negative (RNase P) internal controls were run. The cycle threshold ($C_T$) values of these controls were continuously monitored, and the standard deviation (SD) and coefficient of variation (CV) were monitored. The accumulative SD and CV for each of the internal controls were always below 1% and 2%, respectively. Moreover, for each nucleic acid-extracted patient sample, the RNase P internal control was amplified in a different well, as recommended by the CDC protocol (10). The LDA's running conditions were 48°C for 30 min, 95°C for 10 min, and 50 cycles of 95°C for 15 s and 60°C for 1 min. A sample was considered positive if positive target signals were produced by the ABI 7500 software before a $C_T$ of 40. All samples with $C_T$ results above 40 were considered negative if the RNase P internal control gave a positive signal.

For the Simplexa Flu A/B & RSV assay, a 2-μl heat-treated patient sample was used to run the Simplexa Flu A/B & RSV kit in the 3M integrated cycler in a 10-μl total reaction mixture according to the manufacturer's instructions. The reaction mix components included proprietary concentrations of the DNA polymerase, reverse transcriptase, buffers, deoxynucleoside triphosphates, and bifunctional fluorescent probe-primers. The bifunctional fluorescent probe-primers are used together with the reverse primers to amplify a specific target for influenza A virus (matrix gene), influenza B virus (matrix gene), RSV (matrix gene), and the internal control. Different fluorophore labels were incorporated for the different gene targets: 6-carboxyfluorescein (FAM) for influenza A virus, JOE (6-carboxy-4′,5′-dichloro-2′,7′-dimethoxyfluorescein) for influenza B virus, CFR610 for RSV, and Q670 for the internal control.

A sample was considered positive if a signal was obtained below a $C_T$ value of 40, regardless of the result of the internal control. A sample was considered negative in the absence of a signal and if the internal control was positive. A sample was considered uninterpretable if no signal was obtained from the sample and the internal control did not give a positive signal. Uninterpretable results could be due to qRT-PCR inhibitors.

Of the 170 samples tested by both methods, 78 (45.9%) patient samples were positive for one or more of the viruses tested by the LDA, while 74 (43.5%) patient samples were positive by the Simplexa Flu A/B & RSV kit. The LDA detected 3 patient samples with double infection (1 influenza A virus/RSV and 2 influenza B virus/RSV), while the Simplexa Flu A/B & RSV kit detected the same 3 dually infected patients detected by the LDA and an additional patient sample with influenza B virus/RSV. Four positive patient samples (2 positive for influenza A virus and 2 positive for RSV) tested positive upon initial and repeat testing by the LDA but were negative by the Simplexa Flu A/B & RSV kit. On the other hand, one patient sample was positive upon initial and repeat testing for influenza B virus in the Simplexa Flu A/B & RSV kit but was negative upon initial and repeat testing in the LDA. Thus, the overall sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the Simplexa Flu A/B & RSV kit for detection of influenza A and B viruses and RSV were, 95.1%, 99.6%, 98.7%, and 98.6%, respectively (Table 1). Of importance was the RNase P internal control of the LDA, which gave a positive signal for all the samples tested, while the internal control of the Simplexa Flu A/B & RSV kit failed in 4 patient samples (2.3%) that were negative for influenza viruses and RSV. The Simplexa Flu A/B & RSV kit failure rate was higher than the 1.5% reported for other fully automated assays, such as the eSensor respiratory viral panel (12).

Upon stratifying the patient samples by virus type, the LDA detected 30 influenza A viruses, whereas the Simplexa Flu A/B & RSV kit detected 28. Thus, the sensitivity, specificity, PPV, and NPV of the Simplexa Flu A/B & RSV kit for detection of influenza A virus were 93.3%, 100%, 100%, and 97.9%, respectively (Table 1). The two discrepant samples were weakly positive by the LDA (TaqMan $C_T$ values, 38 and 34) and negative by the Simplexa Flu A/B & RSV kit. In addition, repeating the LDA using a different set of primers and probes targeting a different region in the influenza virus matrix gene also confirmed the initial positive results (11). Molecular typing of these two weak discrepant samples using H1N1-specific primers obtained from the CDC and our laboratory-designed H3N2 primers sequences (H3 forward primer 5′-ATT GGT TGG GAG GGA ATG-3′, H1 reverse primer 5′-TTG AGT GCT TTT RAG ATC TGC-3′, H1 probe 5′-VIC-TTG GTA CGG TTT CAG GCA TCA-TAMRA-3′, where TAMRA is 6-carboxytetramethylrhodamine) revealed that both were influenza A virus type H3N2.

The LDA detected influenza B virus in 30 patient samples, while the Simplexa Flu A/B & RSV kit detected the virus in 31 patient samples. On the basis of these results, the Simplexa Flu A/B & RSV kit's sensitivity, specificity, PPV, and NPV were 100%, 98.9%, 96.8%, and 100%, respectively (Table 1). The one discrep-

**TABLE 2** Comparison between $C_T$ values obtained from testing reference and clinical isolates by LDA and Focus Simplexa FluA/B and RSV kit

| Virus | $C_T$ value | | | | | |
|---|---|---|---|---|---|---|
| | Influenza A virus | | Influenza B virus | | RSV | |
| | LDA | Simplexa | LDA | Simplexa | LDA | Simplexa |
| A/Brisbane/59/07 (H1N1) | 34.2 | 32.2 | | | | |
| A/California/7/09 pdm (H1N1) | 17 | 17.6 | | | | |
| A/New Caledonia/20/99 (H1N1) | 17.3 | 16.7 | | | | |
| A/Perth/16/09 (H3N2) | 18.1 | 15.4 | | | | |
| A/Puerto Rico/8/34 (H1N1) | 17.7 | 16.2 | | | | |
| A/Solomon Islands/3/06 (H1N1) | 16.6 | 14.1 | | | | |
| A/Wisconsin/67/05 (H3N2) | 20.1 | 15.5 | | | | |
| B/Brisbane/60/08 | | | 15.8 | 18.9 | | |
| B/Florida/4/06 | | | 27.1 | 27.2 | | |
| B/Shangdon/7/97 | | | 14.8 | 16 | | |
| RSV-A (ATCC VR-1302) | | | | | 19.8 | 20.6 |
| RSV-A (clinical sample) | | | | | 23.8 | 24.3 |
| RSV-B (clinical sample) | | | | | 26.2 | 26 |
| RSV-B (clinical sample) | | | | | 23 | 20.7 |

ant influenza B virus-positive specimen was also positive for RSV. Furthermore, analysis of this patient sample using a different set of primers and probe published by the CDC showed that it was also negative for influenza B virus (10). This could imply that either the Simplexa Flu A/B & RSV kit gave a false-positive influenza B virus result or the assay's sensitivity to detect the influenza B virus genome is greater than that of both our LDA and the CDC influenza B virus assay.

Conversely, the LDA detected 21 patient samples with RSV, while the Simplexa Flu A/B & RSV kit detected 19 patient samples with RSV. Thus, the Simplexa Flu A/B & RSV kit sensitivity, specificity, PPV, and NPV were 95.1%, 99.6%, 98.7%, and 98.6%, respectively (Table 1). Tests for two discrepant samples that were positive by the LDA (TaqMan $C_T$ values, 38.1 and 34.8) and negative by the Simplexa Flu A/B & RSV kit were repeated twice with similar results. In addition, the two discrepant RSV-positive samples were typed as RSV group B using the sets of primers and probes reported by Hu et al., where the RSV group A probe was labeled with FAM, while the RSV group B probe was labeled with VIC (9).

In our hands, the Simplexa Flu A/B & RSV kit performance using the modified protocol was different from that described in a recent report by Alby et al., who evaluated the kit on viral RNA-extracted samples (13). The reported sensitivities for influenza A and B viruses and RSV were 82.8%, 76.2%, and 94.6%, respectively. The lower sensitivity was noted not only when the authors compared the Simplexa Flu A/B & RSV kit to their LDA but also when they compared it to the FDA-cleared Nanosphere Verigene Respiratory Virus Plus test (Northbrook, IL). We hypothesize that the lower sensitivity noted by Alby et al. (13) could be in part due to the presence of an interfering substance in the extraction step that specifically interfered with the Simplexa Flu A/B & RSV kit performance. This hypothesis was not raised by the authors. On the other hand, the higher sensitivity that was noted for the Simplexa Flu A/B & RSV kit in our study could be in part due to better PCR amplification efficiency as a result of the direct utilization of a small sample volume of 2 μl, which can minimize the presence of any qRT-PCR-interfering substances. In addition, the type of viral strains circulating in Israel could explain the preferential amplification compared to that of the strains evaluated in the study of Alby et al. (13).

The analytical sensitivity of the Simplexa Flu A/B & RSV kit was investigated on serially diluted complete viruses and extracted RNA. This was performed in order to determine whether the discrepant results were due to a reduced analytical sensitivity of the Simplexa Flu A/B & RSV kit. Serial dilution of the influenza A virus A/Puerto Rico/8/34 (H1N1) repeatedly (three times) revealed that the Simplexa Flu A/B & RSV kit was about 1 log unit (10-fold) more sensitive that the LDA. The Simplexa Flu A/B & RSV kit detected 0.001 PFU of A/Puerto Rico/8/34 (H1N1), while the LDA detected 0.08 PFU. Similarly, the Simplexa Flu A/B & RSV kit detected 1 log unit more of the serially diluted RSV-A (ATCC VR-1302) than the LDA. The Simplexa Flu A/B & RSV kit and the LDA detected 0.0003 and 0.001 RSV-A 50% tissue culture infective doses, respectively. On the other hand, the LDA detected about 1.5 more log units of diluted influenza B/Lee/40 virus than the Simplexa Flu A/B & RSV kit. The LDA detected 1.6 50% chicken embryo infectious doses ($CEID_{50}$s) of influenza B/Lee/40 virus, while the 3M Simplexa Flu A/B & RSV kit detected 63 $CEID_{50}$s. Thus, the reduced clinical sensitivity does not appear to be due to reduced analytical sensitivity. On the contrary, the Simplexa Flu A/B & RSV kit appears to be more sensitive than the LDA with the virus strains evaluated.

In order to further evaluate the versatility of the 3M Simplexa Flu A/B & RSV kit for the detection of different influenza virus (A and B) and RSV strains, several influenza virus strains [A/New Caledonia/20/99 (H1N1), A/Puerto Rico/8/1934 (H1N1), A/California/7/09 pdm (H1N1), A/Perth/16/09 (H3N2), A/Wisconsin/67/05 (H3N2), A/Solomon Islands/3/06 (H1N1), A/Brisbane/59/07 (H1N1), B/Florida/4/06, B/Shangdon/7/97, B/Brisbane/60/08] and RSV-A (ATCC VR-1302) and RSV group A and B strains were evaluated in duplicate by both methods. The 3M Simplexa Flu A/B & RSV kit gave $C_T$ values comparable to those provided by the LDA for the detection of these strains (Table 2).

To our knowledge, this is the first report describing an evaluation of the modified protocol to run the Simplexa Flu A/B & RSV kit on the 96-well universal disc without extracting viral nucleic acid. The comparable sensitivity and specificity of the Simplexa

Flu A/B & RSV kit to those of the LDA make it an excellent alternative to the assay that requires nucleic acid extraction. The implementation of the described modified protocol for the Simplexa Flu A/B & RSV kit will reduce the turnaround time for obtaining the results from 6 h in our LDA to 1.5 h, thus making is an attractive assay, in particular, when the result will impact patient management. Furthermore, from our experience, deleting the extraction step will allow laboratories to reduce the cost of running the test by eliminating the direct and indirect costs of running the extraction step.

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