Evaluation of an Internally Controlled Assay to Detect Influenza A, Influenza B, and RSV Using the 3M Integrated Cycler

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Revised Abstract

Introduction: The recent influenza (flu) pandemic has underscored a need for rapid molecular tests for detection of respiratory viruses associated with flu-like symptoms. This study evaluated the performance of a multiplex assay designed to detect and discriminate between influenza A, influenza B, and respiratory syncytial virus (RSV) (Simplexa™ Flu A/B & RSV assay, Focus Diagnostics, Cypress, CA). The assay was performed on the 3M Integrated Cycler microfluidic platform, and included a novel RNA internal extraction and amplification control, along with a stabilized positive control comprising all three viruses.

Methods: A panel of influenza A, influenza B, and RSV positive and negative clinical respiratory specimens was collected. Viral RNA from these specimens was extracted using both the MagNA Pure LC® and NucliSENS® EasyMag™ instruments, and was amplified using the 3M Integrated Cycler real-time PCR instrument. Analytical sensitivity was determined using panels of spiked specimens, and specificity was assessed by testing multiple influenza and RSV strains, along with other respiratory viruses. The multiplex RT-PCR assay's positive control was created using inactivated influenza A and B and RSV viruses. Accelerated stability studies for the positive control materials were conducted by incubating reagents at 28°C, and monitoring for deterioration of viral RNA.

Results: The multiplexed PCR assay showed 100% positive agreement with all clinical samples that were positive by culture or direct fluorescent antibody (DFA) assay. Positive PCR results were observed for some specimens that were reported negative by culture or DFA, which indicate a potentially higher sensitivity for PCR. The analytical sensitivity for all targets was <30 $TCID_{50}$/mL, and results were consistent between both extraction methods. Specificity studies showed no cross reactivity with other respiratory pathogens, and the assay had the ability to detected all circulating influenza A subtypes tested (including the pandemic H1N1 strain), along with multiple influenza B, RSV-A and RSV-B strains. The positive control was able to detect all targets in a single well. The accelerated stability studies indicated that the controls would be stable for >2 years storage at -20° C, based on accelerated stability studies.

Conclusion: These preliminary data are very encouraging. The Simplexa assay performed well and PCR results from 96 reactions were available in <70 minutes. The use of a multiplexed positive control reduces the number of controls required per run, and the inclusion of an extraction and amplification internal control provides the ability to monitor assay performance in each individual specimen. These features would make the assay a good choice for rapid molecular testing.

Methods

Virus strains. The following virus strains were tested: **Influenza A:** Influenza A/PR/8/34 H1N1, $10^{9.5}$ $TCID_{50}$/mL (Advanced Biotechnologies, Inc., Columbia, MD), Influenza A/Hong Kong/8/68 H3N2, $10^{7.5}$ $TCID_{50}$/mL (ABI). **Influenza B:** Influenza B/Malaysia/2506/2004, 5.16×10^{8} $TCID_{50}$/mL (Virapur, San Diego, CA), Influenza B/Great Lakes/1739/54, 1.58×10^{7} $TCID_{50}$/mL (ATCC, Manassas, VA), **RSV:** RSV A1, 3.2×10^{5} $TCID_{50}$/mL (Virapur), RSV B2, 2.8×10^{5} $TCID_{50}$/mL (ATCC).

Sample preparation. 200 µL of each clinical specimen was extracted using the Roche MagNA Pure LC automated system with a Total Nucleic Acid Isolation Kit (Roche Diagnostics, Indianapolis, IN), and eluted in 50 µL of elution buffer. The same clinical specimens (200 µL of sample input) were extracted using a NucliSENS easyMAG (bioMerieux, Durham, NC), and eluted with 50 µL. A RNA internal control (Focus Diagnostics, Inc.) was added to each specimen and control prior to extraction to monitor the extraction process and detect PCR inhibition.

Primers. Primers were designed to target a conserved region of the influenza A matrix segment, influenza B matrix segment and RSV M gene. An additional set of primers was used to detect the RNA internal control. Each probe was labeled with a different fluorescent dye with a distinct emission profile to distinguish the fluorescent signal from each target or internal control.

Multiplex Real-time PCR amplification and detection. A one-step RT-PCR assay was carried out with 5 µL of extracted RNA and 5 µL of reaction mix (Simplexa Flu A/B & RSV assay, Focus Diagnostics, Inc.). RT-PCR was performed by using an Integrated Cycler microfluidic platform (3M, St. Paul, MN) programmed with the following parameters: 1 cycle of 47°C for 10 min; 1 cycle of 97°C for 2 min; 45 cycles of 97°C for 5 sec and 58°C for 30 sec. Fluorescent signal for target-specific amplification was detected at 58°C.

Methods (Cont.)

Analytical specificity: Genomic DNA or RNA of a variety of viral (≥10^5 $TCID_{50}$/mL) and bacterial (≥10^6 CFU/mL) pathogens, or clinical specimens with Ct ≤ 30 for each targeted pathogen were tested to verify lack of cross-reactivity of the Simplexa assay with nucleic acids from other organisms. For each contrived sample, organisms were spiked into a 200 µL aliquot of Universal Transport Medium (UTM) (Diagnostic Hybrids, Athens, OH).

Analytical sensitivity: Influenza A , influenza B and RSV strains were serially diluted in UTM and extracted in 10 replicates. Each extracted sample was tested in duplicate wells for a limit of detection (LoD) study. Extracted nucleic acid samples from a defined $TCID_{50}$/mL concentration of each virus strain were tested with the Simplexa assay.

Clinical specimens: 239 de-identified residual clinical specimens (Table 1) were extracted with the 2 automated systems described above, and the Simplexa multiplex PCR assay results were compared with previous DFA, culture or RT-PCR results. These specimens included nasal swabs, nasopharyngeal swabs, and swabs with unspecified respiratory sources. To eliminate bias, operators were blinded to previously reported results. Simplexa assay results were compared to previous results for concordance. Any specimens with discrepant results were tested at Focus Diagnostics reference laboratory for additional resolution using Flu A/B or RSV RT-PCR testing.

Table 1. Clinical Specimen Summary

Specimen Type	Culture	DFA	RT-PCR	Total
Flu A Positive	18	21	0	39
Flu B Positive	11	23	22	56
Flu A/B Negative	0	0	51	51
RSV Positive	0	0	55	55
RSV Negative	0	0	38	38
Total # of Clinical Specimens				239

Accelerated Stability Studies: Positive control and RNA internal control were aliquoted into 5 vials. One vial was stored at -20° C as the 0 day control. The other 4 vials were stored at 28° C for 1, 2, 3, and 4 days, respectively, followed by storage at -20° C. After completion of the process, all positive controls and RNA internal control were extracted with the MagNA Pure LC system and tested in quadruplicate reactions with the Simplexa multiplexed PCR assay.

Results

Specificity Studies: The Simplexa multiplexed PCR assay did not detect any of the pathogens listed in Table 2 and was specific for the targeted viruses. In addition, NCBI Genbank database searches indicated that the targeted regions did not have significant homology with sequences from other pathogens.

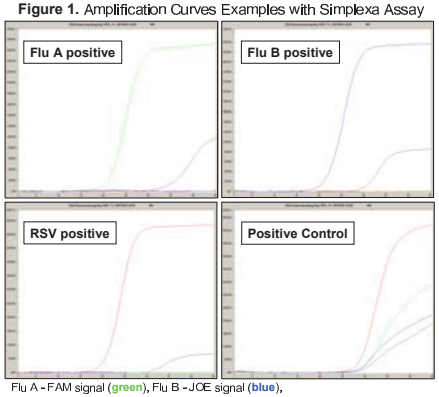
Analytical Sensitivity: The LoD, defined as the lowest concentration with >95% detection, was determined to be <5 $TCID_{50}$/mL for influenza A, <30 $TCID_{50}$/mL for influenza B, and <1 $TCID_{50}$/mL for RSV (Table 3).

Accelerated Stability Studies: The Simplexa positive control and RNA internal control demonstrated >2 years stability at -20° C, based on storage at 28° C and Arrhenius equation (Table 4).

Method Comparison Studies: The Simplexa multiplexed PCR assay provided results with >95% positive and negative agreements compared to previous clinical results for influenza A, B and RSV (Tables 5-7).

Results (Cont.)

Figure 1. Amplification Curves Examples with Simplexa Assay



Flu A - FAM signal (green), Flu B - JOE signal (blue),
RSV - CFR610 signal (red), RNA Internal Control - Quasar 670 signal (purple)

Table 2. Specificity Testing with Different Respiratory Pathogens

Adenovirus 1A	*Haemophilus influenzae*	Parainfluenza type-1
Adenovirus 7A	*Lactobacillus plantarum*	Parainfluenza type-2
Bordetella pertussis	*Legionella longbeachae*	Parainfluenza type-3
Chlamydophila pneumoniae	Measles	*Pseudomonas aeruginosa*
Coronavirus 229E	Human Metapneumovirus	Rhinovirus A1
Coronavirus OC43	*Moraxella catarrhalis*	*Staphylococcus aureus*
Corynebacterium diphtheriae	Mumps	*Staphylococcus epidermidis*
Cytomegalovirus	*Mycobacterium tuberculosis*	*Streptococcus pneumoniae*
Enterovirus 71	*Mycoplasma pneumoniae*	*Streptococcus pyogenes*
Epstein Barr Virus	*Neisseria elongata*	*Streptococcus salivarius*
Escherichia coli O157H7	*Neisseria meningitides*	

Table 3. Analytical Sensitivity, Limit of Detection

Influenza A	Extraction Method	$TCID_{50}$/mL	Average Ct (FAM)	Min Ct	Max Ct	Replicates Detected
Influenza A/PR/8/34 (H1N1)	easyMAG	3.16	35.52	35.00	36.40	19/20
	MagNA Pure	3.16	37.22	35.80	42.40	19/20
Influenza A/Hong Kong/8/68 (H3N2)	easyMAG	0.63	34.43	33.60	35.40	20/20
	MagNA Pure	0.63	33.62	30.00	36.80	20/20
Influenza B	**Extraction Method**	**$TCID_{50}$/mL**	**Average Ct (JOE)**	**Min Ct**	**Max Ct**	**Replicates Detected**
Influenza B/Malaysia/2506/2004	easyMAG	25.8	34.95	34.20	35.60	20/20
	MagNA Pure	25.8	35.25	34.20	36.50	20/20
Influenza B/Great Lakes/1739/54	easyMAG	15.8	36.04	35.00	37.2	19/20
	MagNA Pure	15.8	35.94	35.10	39.2	20/20
RSV	**Extraction Method**	**$TCID_{50}$/mL**	**Average Ct (CFR610)**	**Min Ct**	**Max Ct**	**Replicates Detected**
RSV A2	easyMAG	0.32	34.33	33.60	35.30	20/20
	MagNA Pure	0.32	35.27	34.50	36.90	20/20
RSV B1	easyMAG	0.70	33.03	30.00	35.10	20/20
	MagNA Pure	0.70	33.75	32.10	36.60	20/20

Fluorescence Thresholds: Flu A (FAM) = 15,000, Flu B (JOE) = 10,000, RSV (CFR610) = 25,000

Table 4. Positive Control and RNA Internal Control Stability

Storage at 28° C	Equivalent to Storage at -20° C	Flu A		Flu B		RSV		RNA IC	
		Ave. Ct	%Δ Ct	Ave. Ct	% ΔCt	Ave. Ct	% ΔCt	Ave. Ct	%ΔCt
0 Day	0 Months	29.8	0.0%	29.0	0.0%	30.3	0.0%	30.3	0.0%
1 Day	7 Months	30.3	1.8%	29.3	1.2%	29.7	-1.9%	30.8	1.6%
2 Days	13 Months	32.2	8.2%	31.8	9.9%	30.5	0.6%	30.6	0.9%
3 Days	20 Months	32.3	8.5%	32.2	11.1%	30.6	0.8%	30.7	1.2%
4 Days	26 Months	32.6	9.5%	33.2	14.8%	30.6	0.8%	31.0	2.2%

Each positive control and RNA internal control time point was extracted by MagNA Pure system and tested in quadruplicate with the Simplexa Flu A/B & RSV Real-Time RT-PCR assay. The average of 4 reactions and % change in cycle threshold (% ΔCt compared to 0 day) were calculated. Equivalent storage at -20° C was based on Arrhenius equation.

Results (Cont.)

Table 5. Simplexa Concordance for Influenza A

		Previous Results			% Agreement
		Flu A Positive	Flu A Negative	Total	
Simplexa Flu A/B & RSV Real-Time RT-PCR	Flu A Positive	38	2*	40	% Positive Agreement 97.4% (38/39) 95% CI: 86.8-99.5
	Flu A Negative	1**	198	199	% Negative Agreement 99.0% (198/200) 95% CI: 96.4-99.7
	Total	39	200	239	

*Samples 41 and 75 were detected as flu A positive by Simplexa assay, whereas previous PCR result detected these samples as flu A negative. The Simplexa assay results were identical for both samples by both extraction methods. Samples 41 and 75 were confirmed to be flu A positive by Focus Diagnostics reference laboratory RT-PCR.
** Sample 111 was detected as flu A negative by Simplexa assay, whereas previous DFA result detected as flu A positive. The Simplexa assay results were identical for both extraction methods. Sample 111 was confirmed to be flu A positive by Focus Diagnostics reference laboratory RT-PCR.

Table 6. Simplexa Concordance for Influenza B

		Previous Results			% Agreement
		Flu B Positive	Flu B Negative	Total	
Simplexa Flu A/B & RSV Real-Time RT-PCR	Flu B Positive	55	1*	56	% Positive Agreement 98.2% (55/56) 95% CI: 90.6-99.7
	Flu B Negative	1**	182	183	% Negative Agreement 99.5% (182/183) 95% CI: 97.0-99.9
	Total	56	183	239	

*Sample 209 was detected as flu B positive by the Simplexa assay, whereas the previous PCR result was negative for flu B. The Simplexa assay results were identical for both extraction methods. Focus Diagnostics reference laboratory RT-PCR detected sample 209 as negative for flu B.
** Sample 12 was detected as negative for flu B by the Simplexa assay, whereas previous culture results determined the sample to be flu B positive. Simplexa assay results were identical for both extraction methods. Focus Diagnostics reference laboratory RT-PCR confirmed sample 12 to be negative for flu B.

Table 7. Simplexa Concordance for RSV

		Previous Results			% Agreement
		RSV Positive	RSV Negative	Total	
Simplexa Flu A/B & RSV Real-Time RT-PCR	RSV Positive	55	1*	56	% Positive Agreement 100% (55/55) 95% CI: 93.5-100
	RSV Negative	0	183	183	% Negative Agreement 99.5% (183/184) 95% CI: 97.0-99.9
	Total	55	184	239	

*Sample 25 was detected as dual positive for flu A and RSV by the Simplexa assay, whereas previous culture result detected only flu A. Simplexa assay results were identical for both extraction methods. Sample 25 was confirmed to be positive for both flu A and RSV by Focus Diagnostics reference laboratory RT-PCR.

Conclusions

- The preliminary data are very encouraging. The Simplexa assay performed well and PCR results from 96 reactions were available in <70 minutes.
- The Simplexa assay's limit of detection for influenza A, influenza B, and RSV was <5 $TCID_{50}$/mL, <30 $TCID_{50}$/mL, and <1 $TCID_{50}$/mL, respectively, with both automated extraction systems.
- Positive and negative agreement with previous clinical results demonstrated that the Simplexa Flu A/B & RSV assay could effectively detect seasonal influenza A, B and RSV.
- The use of a multiplexed positive control reduces the number of controls required per run, and the inclusion of an extraction and amplification internal control provides the ability to monitor assay performance in each individual specimen. These features would make the assay a good choice as a rapid molecular test.
- Simplexa Flu A/B & RSV assay is in development, it is not currently available for sale, and is not FDA cleared.

CE Mark pending

FOCUS Diagnostics

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