Detection and Discrimination of Influenza A, B, and Respiratory Syncytial Viruses using the 3M Integrated Cycler

Michael Aye*, Jules Chen, Lakshmi Nair, Yuan Xie, Huong Mai, Shannon Dempsey, Yin-Peng Chen, and Maurice Exner

Focus Diagnostics Inc., Cypress, CA

Abstract

Objective: Recently, it has become evident that many rapid tests designed to detect respiratory viruses lack sensitivity. As a result, a clinical need has arisen for more sensitive tests that will detect and discriminate influenza viruses. Molecular techniques have been shown to be more sensitive than culture or rapid lateral flow methodologies. Therefore, a multiplex RT-PCR assay was designed to detect Influenza A viruses (of all hemagglutinin and neuraminidase types), Influenza B viruses and Respiratory Syncytial virus (RSV).

Methods: Primers were designed to target conserved regions of Influenza A, B, and RSV. Specificity of the assay was tested by running a panel of known influenza strains and against a set of clinical specimens that were verified to be positive for the target viruses. In addition, a panel of other respiratory specimens was tested to determine whether there was any cross reactivity with these organisms. Assay sensitivity was determined analytically by limit of detection studies, and sensitivity was compared to culture, DFA, and RT-PCR methods.

Results: The assay was able to detect multiple influenza types and strains including seasonal H1 and H3 strains, H1N1 (2009), and H5N1. All influenza B strains tested were detected, as were all strains of RSV-A and RSV-B that were tested. The assay was shown to be more sensitive than culture and DFA for all viruses.

Conclusions: The use of this assay on the 3M integrated cycler provides a sensitive, rapid and high throughput method for detecting and discriminating Influenza A, B, and RSV viruses. The portability and ease of use of the instrument make it useful as a first line tool for diagnosis of respiratory infections.

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. An RNA internal control (Focus Diagnostics, Inc.) was added to each specimen and control prior to extraction to monitor the extraction process and to 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.

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.

Methods (Cont.)

Fluorescent signal for target-specific amplification was detected at 58˚C.

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 ten 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 clinical specimens (Table 1) were extracted with two automated systems described above, and the Simplexa™ 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, previously reported results were blinded to operators. Simplexa™ results were compared to previous results for concordance. Any discrepant specimens were tested by the 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

Results

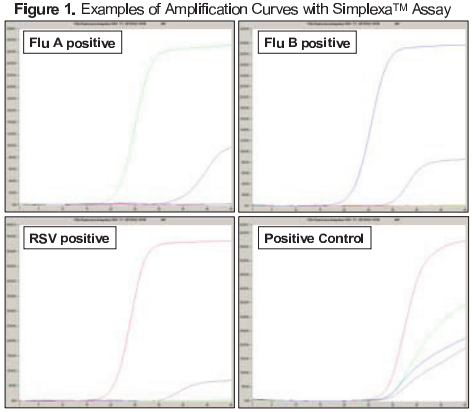
Specificity Studies: The Simplexa™ assay did not detect any of the pathogens listed in Table 2 and was specific for the targeted viruses. In addition, database searches indicated that the targeted regions did not have significant homology with sequences from other pathogens.

Limit of Detection: 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).

Method Comparison Studies: As shown in Tables 4-6, the Simplexa™ assay provided results with >95% positive and negative agreements compared to previous clinical results for Influenza A, B and RSV.

Results (Cont.)

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

Table 3. Analytical Sensitivity, Limit of Detection studies

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

Results (Cont.)

Table 4. Simplexa™ Concordance for Flu A

		Previous Results: Flu A Positive	Previous Results: Flu A Negative	Previous Results: Total	% Agreement
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. Simplexa™ results were identical for both samples by both extraction methods. Samples 41 and 75 were confirmed to be Flu A positive by Focus reference laboratory.
** Sample 111 was detected as Flu A negative by Simplexa™ assay, whereas previous DFA result detected as Flu A positive. Simplexa™ results were identical for both extraction methods. Sample 111 was confirmed to be Flu A positive by Focus reference laboratory.

Table 5. Simplexa™ Concordance for Flu B

		Previous Results: Flu B Positive	Previous Results: Flu B Negative	Previous Results: Total	% Agreement
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 results detected as Flu B negative. Simplexa™ results were identical for both extraction methods. Focus reference laboratory detected sample 209 as Flu B negative.
** Sample 12 was detected as Flu B negative by the Simplexa™ assay, whereas previous culture results determined the sample to be Flu B positive. Simplexa™ results were identical for both extraction methods. Sample 12 was confirmed to be Flu B negative by Focus reference laboratory.

Table 6. Simplexa™ Concordance for RSV

		Previous Results: RSV Positive	Previous Results: RSV Negative	Previous Results: Total	% Agreement
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 Flu A and RSV dual positive by the Simplexa™ assay, whereas previous culture results detected only Flu A. Simplexa™ results were identical for both extraction methods. Sample 25 was confirmed to be Flu A and RSV dual positive by Focus reference laboratory.

Conclusions

- The Simplexa™ assay's Limit of detection (LoD) 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 Simplexa™ assay, using the 3M Integrated Cycler, provides robust and sensitive detection for Influenza A and B, and RSV, and the features of the instrument (small footprint, high-throughput and rapid cycling) provides an effective option for clinical laboratory testing.

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