Evaluation of Focus Diagnostics Simplexa™ Flu A/B & RSV Direct Real-time PCR assay

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Abstract

Introduction: Influenza A (Flu A), influenza B (Flu B) and respiratory syncytial virus (RSV) are clinically important viral pathogens that cause respiratory illness in millions of individuals worldwide each year. The Simplexa™ Flu A/B & RSV Direct assay is in development for differential detection of Flu A, Flu B and RSV from nasopharyngeal swabs (NPS) without extraction. The goal of this study was to compare the performance of the Simplexa Flu A/B & RSV Direct assay to culture and to a FDA-cleared real-time PCR methodology that uses automated nucleic acid extraction.

Materials and Methods: Performance of the Simplexa Flu A/B & RSV Direct assay was assessed with panels of clinical NPS specimens, and the results were compared to previous culture and/or qualitative real-time PCR results. Specificity studies were performed using a contrived panel of 31 respiratory pathogens and normal flora. Limit of detection (LoD) studies were performed using serially diluted viral stocks, and the LoD was determined as the lowest concentration where ≥95 % replicates were detected. In addition, assay inhibition/interference was determined by spiking relevant concentrations of potentially interfering substances.

Results: The Simplexa Flu A/B & RSV Direct assay showed 100% agreement for all clinical specimens that were reported positive by both culture and the comparator real-time PCR assay. Negative agreement for Flu A, Flu B and RSV were 98.4% (186/189), 99.5% (187/188) and 100% (202/202) respectively. The multiplexed assay was specific and no cross-reactivity was observed with other organisms. The assay detected geographically diverse strains of Flu A including the seasonal H1 and H3 subtypes and the 2009 pandemic H1N1 strain. It was also capable of detecting all tested strains of Flu B, RSV-A and RSV-B, and the analytical sensitivity for each of the three targets was less than 30 $TCID_{50}$/mL. Studies using clinically relevant interferents did not show any inhibition of target detection.

Conclusion: The sample-to-answer Simplexa Flu A/B & RSV Direct assay can provide results from 8 samples in approximately 1 hour with minimal hands-on manipulation. The assay displayed similar performance to conventional detection methods, including a real-time PCR method that requires nucleic acid extraction.

Methods

Virus strains: LoD for the Simplexa Flu A/B & RSV Direct assay was determined by testing limiting dilutions of the following viral stocks: **Influenza A**: Influenza A/PR/8/34 H1N1 (1.0 x $10^{5.23}$ $TCID_{50}$/mL) and Influenza A/Hong Kong/8/68 H3N2 (1.0 x $10^{6.68}$ $TCID_{50}$/mL). **Influenza B**: Influenza B/Malaysia/2506/2004 (1.26 x 10^{6} $TCID_{50}$/mL) and Influenza B/Great Lakes/1739/54 (2.45 x 10^{5} $TCID_{50}$/mL). **RSV**: RSV A2 (3.2 x 10^{5} $TCID_{50}$/mL) and RSV B CH93-18(18) (1.0 x $10^{6.18}$ $TCID_{50}$/mL).

Real-time RT-PCR Amplification and Detection: Simplexa Direct detection was performed on the Direct Amplification Disc (DAD) with 50 µL of sample loaded into the sample port and 50 µL of Simplexa Flu A/B & RSV Direct Reaction Mix loaded into the reaction port. All testing was performed on the 3M Integrated Cycler instrument (3M, St. Paul, MN). Data collection and analysis were performed with Integrated Cycler Studio software.

Specificity and analytical reactivity: The cross-reactivity panel was contrived by spiking 10^{6} CFU/mL of bacteria or 10^{5} $TCID_{50}$/mL of virus in pooled negative swab. Analytical reactivity was tested with $TCID_{50}$/mL of 10^{3} or lower for Flu A, Flu B and RSV strains (Table 3).

Interference: The interference panel was contrived by spiking Flu A, Flu B or RSV at 2-4 fold of the LoD concentration in negative swab containing each interfering substance as listed in Table 5. A control sample with no interferent added was also tested for each target analyte.

Methods (Cont.)

Clinical specimens: A panel of 262 nasopharyngeal swab specimens (Table 1) previously tested with culture or conventional RT-PCR that requires nucleic acid extraction were used. Simplexa Flu A/B & RSV Direct results was compared with previously reported results to determine positive and negative agreements. All discordant specimens were re-tested with the Direct assay and with FDA-cleared Simplexa Flu A/B & RSV assay that requires extraction (Focus Diagnostics).

Table 1. Summary of Clinical specimens

Previous Result	Culture	RT-PCR	Total
Flu A Positive	73	0	73
Flu B Positive	24	50	74
RSV Positive	60	0	60
Negative	55	0	55
Total # of Clinical Specimens			262

Results

Limit of Detection: The LoD was <10 $TCID_{50}$/mL for Flu A, <20 for Flu B and <4 for RSV (Table 2).

Specificity: The assay did not show cross-reactivity to the organisms listed in Table 4. Additionally, sequence homology searches did not show significant matches to any of the organisms tested in the cross-reactivity panel.

Method Comparison: The Positive agreements of the results between the Simplexa Direct assay and previous culture or RT-PCR results was 100% for all three analytes; negative agreements was 98.4% for Flu A, 99.5% for Flu B and 100% for RSV.

Table 2. Limit of Detection

Influenza A	$TCID_{50}$/mL	Average Ct (FAM)	Min Ct	Max Ct	Replicates Detected
Influenza A/PR/8/34 (H1N1)	$1X10^{-2}$	35.9	34.3	37.6	20/20
Influenza A/Hong Kong/8/68 (H3N2)	$1X10^{1}$	37.7	36.0	39.0	20/20
Influenza B	**$TCID_{50}$/mL**	**Average Ct (JOE)**	**Min Ct**	**Max Ct**	**Replicates Detected**
Influenza B/Great Lakes/1739/54	$2X10^{0}$	35.8	35.0	36.3	20/20
Influenza B/ Malaysia/2506/2004	$2X10^{1}$	36.3	35.1	37.4	20/20
RSV	**$TCID_{50}$/mL**	**Average Ct (CFR610)**	**Min Ct**	**Max Ct**	**Replicates Detected**
RSV A2	$3X10^{0}$	36.5	34.8	38.3	20/20
RSV B CH93-18(18)	$4X10^{0}$	37.1	36.0	39.7	20/20

Ct = cycle threshold

Results (Cont.)

Table 3. Pathogens in Analytical Reactivity Testing

Influenza A/Solomon Island/03/06 H1	Influenza B/Panama/45/90
Influenza A/Taiwan/42/06 H1N1	Influenza B/Lee/40
Influenza A/Brisbane/59/07 H1	Influenza B/Allen/45
Influenza A/Swine NY/02/2009 H1	Influenza B/Hong Kong/5/72
Influenza A/WS/33 H1N1	Influenza B/Maryland/1/59
Influenza B/Florida/04/06	Influenza B/Florida/07/04
Influenza A/California/7/2009 NYMC X-179A	Influenza B/Florida/04/06
Influenza A/Port Chalmers/1/73 H3N2	Influenza B/Florida/02/06
Influenza A/Wisconsin/67/05 H3	RSV A-Long
Influenza A/New Caledonia/10/07 H1N1	RSV B-Wash/18537/62
Influenza A/Brisbane/10/07 H3	RSV B-WV/14617/85
Influenza B/Taiwan/2/62	RSV B-9320

Table 4. Organisms used in Specificity Testing

Adenovirus 1	Mumps
Adenovirus 7A	*Mycobacterium tuberculosis*
Bordetella pertussis	*Mycoplasma pneumoniae,* strain M129
Chlamydia pneumoniae	*Neisseria elongata*
Coronavirus 229E	*Neisseria meningitidis*
Corynebacterium diphtheriae	Parainfluenza type 1
Cytomegalovirus	Parainfluenza type 2
Enterovirus 71	Parainfluenza type 3
Epstein Barr Virus	*Pseudomonas aeruginosa*
Escherichia coli, O157H7	Rhinovirus A1
Haemophilus influenzae	*Staphylococcus aureus* , COL
Human metapneumovirus	*Staphylococcus epidermidis*
Lactobacillus plantarum, 17-5	*Streptococcus pneumoniae*
Legionella longbeachae	*Streptococcus pyogenes*
Measles	*Streptococcus salivarius*
Moraxella catarrhalis, Ne 11	

Table 5. List of Potential Interferents

List of Interferents	Concentration tested
Whole blood	2% (v/v)
Afrin (Oxymetazoline)	15% (v/v)
Beconase AQ (Beclomethasone)	5% (v/v)
Fluticasone	5% (v/v)
Zicam nasal gel	5% (v/v)
Mucin	60 µg/mL
Tobramycin	4 µg/mL
Relenza (Zanamivir)	3.3 mg/mL
Mupirocin	6.6 mg/mL
Tamiflu (Oseltamivir)	1.0 µM

Results (Cont.)

Table 6. Simplexa Direct Concordance for Flu A

Simplexa™ \ Previous Results	Flu A Positive	Flu A Negative	Total	% Agreement
Flu A Positive	73	3*	76	% Positive Agreement 100% (73/73)
Flu A Negative	0	186	186	% Negative Agreement 98.4% (186/189)
Total	73	189	262	

* These three specimens were initially detected as influenza A positive with cycle threshold (Ct) values of 38.7, 39.5 and 39.8, but all three gave negative results upon repeat testing.

Table 7. Simplexa Direct Concordance for Flu B

Simplexa™ \ Previous Results	Flu B Positive	Flu B Negative	Total	% Agreement
Flu B Positive	74	1*	75	% Positive Agreement 100% (74/74)
Flu B Negative	0	187	187	% Negative Agreement 99.5% (187/188)
Total	74	188	262	

* This specimen was initially detected as influenza B positive with a Ct of 39.0, but gave a negative result upon repeat testing.

Table 8. Simplexa Direct Concordance for RSV

Simplexa™ \ Previous Results	RSV Positive	RSV Negative	Total	% Agreement
RSV Positive	60	0	60	% Positive Agreement 100% (60/60)
RSV Negative	0	202	202	% Negative Agreement 100% (202/202)
Total	60	202	262	

Conclusions

- The Simplexa Direct assay provides results from 8 samples in about 1 hour with minimal hands-on time.
- The Simplexa Direct assay accurately detected Influenza A, influenza B and RSV from unextracted samples, and demonstrated good clinical agreements with culture or a reference RT-PCR assay that required nucleic acid extraction.

Acknowledgements

Funding for this research and development was provided by the Biomedical Advanced Research and Development authority in the US Department of Health and Human Services under contract HHSO100201000049C.

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