DEVELOPMENT AND VALIDATION OF FRAGMENT AND SEQUENCING ASSAYS AS COMPOSITE COMPARATOR METHOD FOR NxTAG® RESPIRATORY PATHOGEN PANEL

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Background and Objective

Validated sequencing assays are often used as the reference method for clinical performance evaluation of *in-vitro* diagnostic tests. NxTAG® Respiratory Pathogen Panel assay is a qualitative IVD test intended for the simultaneous detection and identification of nucleic acids from 20 respiratory viruses and bacteria. Of the 20 respiratory organisms detected by the assay, sequencing was the comparator for 12 of those targets: Influenza A, Influenza A H1, Coronaviruses 229E, OC43, NL63, and HKU1, Rhinovirus/Enterovirus, Adenovirus, Parainfluenza 4, Human Bocavirus, *Chlamydophila pneumoniae,* and *Mycoplasma pneumoniae*. Due to the large number of targets for which sequencing was the comparator and limitations of sample volume, a two-step composite comparator method was developed and validated (Figure 1). The first step was screening with five different multiplexed fragment assays followed by the second step of bidirectional sequencing for individual targets. Each fragment assay was designed to identify 3 to 4 of the 12 targets. All samples were tested with all five fragment assays to screen for negative clinical samples. Samples that generated a positive result from at least one of the five assays were then subjected to bidirectional sequencing. The bidirectional sequencing result was considered as the final call for those samples. **The objective of this study was to evaluate and validate the performance of the composite comparator method through analytical and clinical studies using xTAG® Respiratory Viral Panel (RVP) as the reference method.**

Materials and Methods

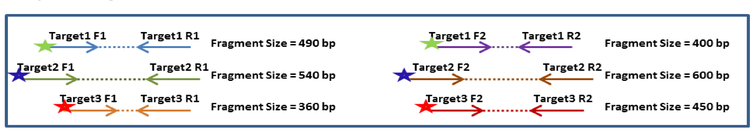
Fragment Analysis

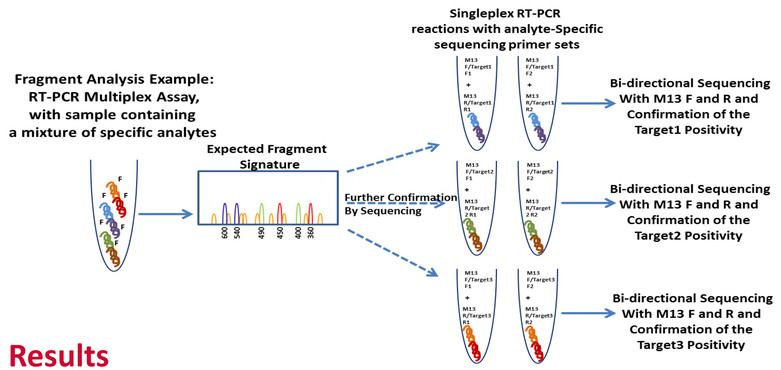
For each of the 12 targets, two independent sets of primers were designed (24 sets in total) for inclusion into the fragment assays. One primer from each set was labelled with an appropriate dye (i.e. FAM™, VIC®, NED™, or PET®) and designed to generate a distinct fluorescently labelled fragment of a specific size through the multiplex RT-PCR reactions of the assay. The fragment size would be an indication of which target existed in a given sample. The intensity of each fluorescent fragment signal was measured in Relative Fluorescent Units (RFU) by GeneMapper® software on the ABI® 3730*xl* DNA Analyzer. A fluorescent DNA ladder (LIZ® dye) added to each sample was used as a size standard.

Bidirectional Sequencing

Two sets of sequencing primers were independently developed and validated for each of the 12 targets using single plex RT-PCR reactions followed by bidirectional Sanger dideoxy sequencing. RT-PCR was performed using the OneStep RT-PCR kit (Qiagen). In brief, 5 µl of nucleic acid extract was used for RT-PCR with M13 tagged analyte specific primers. After completion of RT-PCR, Exonuclease I and Shrimp Alkaline Phosphatase were used to remove unincorporated primers and dNTPs. Dye-labeled terminator cycle sequencing was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (ThermoFisher) according to the manufacturer's instructions. Sample electrophoresis and sequence analysis were performed on the *3730xl* Analyzer (ThermoFisher) using the *3730xl* Data Collection software (v 3.1.1) and Sequencing Analysis software (v 5.4). Sequences that 1) were at least 200 bases in length, 2) had a PHRED score greater than or equal to 20 for at least 90% of the bases, and 3) contained fewer than 5% ambiguous base calls were considered for further analysis using BLAST (NCBI). Acceptable matches to BLAST reference sequences were those with greater than 95% query coverage and identity and an Expected Value (E-Value) less than 10^{-30} when compared to the reference sequence.

Figure 1. Schematic Representation of the Multiplex Fragment Analysis and Sequencing





Results

Analytical Studies

- **Limit of Detection:**. Serial dilutions of 14 representative culture isolates, or clinical samples were tested and generated positive comparator calls for the corresponding targets with at least one fragment and one sequencing assay at a LoD comparable to that of xTAG RVP.
- **Reactivity:** Over 97 strains were tested and generated positive composite comparator calls for the corresponding targets with at least one fragment and one sequencing assay at a LoD and reactivity comparable to that of xTAG RVP.
- **Interference:** In summary, 13 potentially interfering substances and 10 potentially interfering organisms were spiked into 7 multi-analyte sample preparations where 2 – 4 analytes were included in one contrived sample. All targets generated the expected composite comparator positive calls and no interference was observed.
- **Specificity**: Fragment analysis and sequencing primers were analyzed *in silico* against Human (taxid:9606), Bacteria (taxid:2) and Virus (taxid:10239) sequences in GenBank databases. Fragment analysis and sequencing primers did not show any cross-reactivity to any human or respiratory pathogens *in silico*.

Clinical Performance

Table 1. Composite NAAT Coding Algorithm for Clinical Evaluation

Fragment Analysis		Final Fragment Analysis Call	RT-PCR Followed by bidirectional Sequencing		Composite Comparator Call
NAAT#1	NAAT #2		NAAT#1	NAAT #2	
NEG	NEG	NEG	N/A	N/A	NEG
POS	POS	POS	POS	POS	POS
POS	POS	POS	NEG	POS	POS
POS	POS	POS	POS	NEG	POS
POS	POS	POS	NEG	NEG	NEG
POS	NEG	POS	POS	POS	POS
POS	NEG	POS	POS	NEG	POS
POS	NEG	POS	NEG	POS	POS
POS	NEG	POS	NEG	NEG	NEG
NEG	POS	POS	POS	POS	POS
NEG	POS	POS	POS	NEG	POS
NEG	POS	POS	NEG	POS	POS
NEG	POS	POS	NEG	NEG	NEG

Table 2. Summary of Fragment Analysis Call Agreement with xTAG RVP. Final fragment analysis calls on RVP-characterized clinical samples were made as per algorithm in Table 1.

			Positive Agreement				Negative Agreement	
	TP	FN	%	TP / TP + FN	TN	FP	%	TN / TN + FP
Influenza A	77	12	86.5%	(77/89)	702	32	95.6%	(702/734)
Influenza A H1N1	0	0	N/A	(0/0)	714	109	86.8%	(714/823)
Parainfluenza 4	0	1	0.0%	(0/1)	818	0	100.0%	(818/818)
Coronavirus 229E	9	1	90.0%	(9/10)	814	0	100.0%	(814/814)
Coronavirus NL63	11	2	84.6%	(11/13)	809	3	99.6%	(809/812)
Coronavirus OC43	4	0	100.0%	(4/4)	803	14	98.3%	(803/817)
Coronavirus HKU1	10	0	100.0%	(10/10)	806	9	98.9%	(806/815)
Rhinovirus/Enterovirus	112	0	100.0%	(112/112)	671	34	95.2%	(671/705)
Adenovirus	9	1	90.0%	(9/10)	801	9	98.9%	(801/810)

Table 3. Summary of Two-Step Composite Call Agreement with xTAG RVP. Composite fragment analysis followed by bidirectional sequencing calls on RVP-characterized clinical samples were made as per algorithm in Table 1.

			Positive Agreement				Negative Agreement	
	TP	FN	%	TP / TP + FN	TN	FP	%	TN / TN + FP
Influenza A	76	13	85.4%	(76/89)	733	1	99.9%	(733/734)
Influenza A H1N1	0	0	N/A	(0/0)	748	75	90.9%	(748/823)
Parainfluenza 4	0	1	0.0%	(0/1)	818	0	100.0%	(818/818)
Coronavirus 229E	9	1	90.0%	(9/10)	814	0	100.0%	(814/814)
Coronavirus NL63	11	2	84.6%	(11/13)	809	3	99.6%	(809/812)
Coronavirus OC43	4	0	100.0%	(4/4)	809	8	99.0%	(809/817)
Coronavirus HKU1	10	0	100.0%	(10/10)	811	4	99.5%	(811/815)
Rhinovirus/Enterovirus	108	4	96.4%	(108/112)	691	14	98.0%	(691/705)
Adenovirus	9	1	90.0%	(9/10)	809	1	99.9%	(809/810)

In total, 826 xTAG RVP-characterized clinical samples were tested with the fragment analysis panels followed by bidirectional sequencing. The results were compared with xTAG RVP for all the targets listed above with the exception of Influenza A 2009 H1N1, Human Bocavirus, *Chlamydophila pneumoniae,* and *Mycoplasma pneumoniae,* which are not probed by xTAG RVP. In summary, 249 positive calls were made by xTAG RVP. Overall positive agreement between the fragment and sequencing assays and xTAG RVP was 91.2% with an overall negative agreement of 98.5%. Compared to xTAG RVP, 210 additional positive calls were made by the fragment analysis method, of which 106 were confirmed positive by sequencing (data not shown).

Conclusion

The fragment and sequencing assays demonstrated acceptable LoD and reactivity when tested in this study. Overall agreement between the fragment analysis and sequencing with xTAG RVP supported the use of the two-step composite comparator method for the 12 targets during the NxTAG Respiratory Pathogen Panel assay clinical performance evaluation.

NxTAG® Respiratory Pathogen Panel is for In Vitro Diagnostic Use. Products are region specific and may not be approved in some countries/regions. Please contact Luminex at support@luminexcorp.com to obtain the appropriate product information for your country of residence.