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ANALYTICAL VALIDATION OF SANGER SEQUENCING FOR THE RESPIRATORY SYNCYTIAL VIRUS NS2 GENE FROM NASAL SWABS

Nonstructural Protein II (NS2) gene sequencing assay was developed for precise analysis of respiratory syncytial virus (RSV) infection from nasal swabs. This assay demonstrates robustness and utility in both research and clinical environments, producing consistent and reliable sequencing outcomes for the NS2 gene. Sequencing allows for the monitoring of mutations, such as insertions and deletions, which could inhibit the development of effective therapies. Utilizing Sanger Sequencing, the assay offers timely and cost-effective sequencing, crucial for understanding NS2's role in RSV infection.

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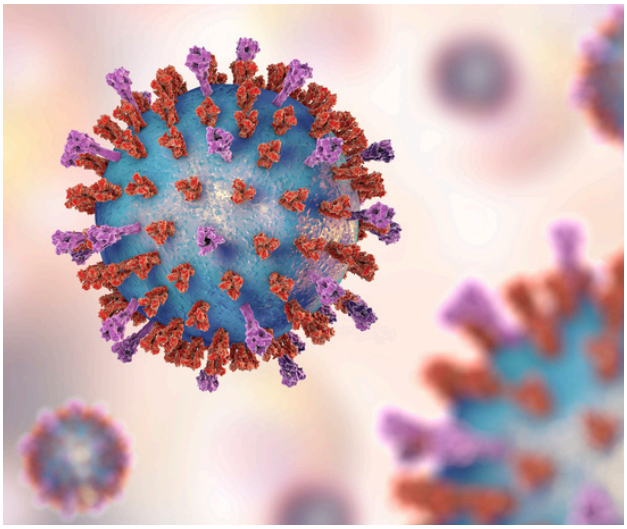
Background

Respiratory syncytial virus (RSV) is the leading cause of lower respiratory tract disease in infants, as well as the leading cause of pneumonia in the elderly and immunocompromised. Nonstructural protein II, also known as NS2, is believed to serve several roles in virus-host interactions including suppression of the host immune system. However, NS2 remains less understood than the similar nonstructural protein I utilized by RSV. Sequencing allows for the monitoring of mutations, such as insertions and deletions, which could inhibit the development of effective therapies. Utilization of Sanger Sequencing allows for less costly and timelier turnaround of sequences, which could prove vital to unraveling the role of NS2 in RSV infection.

Method/Workflow



Nasal swabs were collected from donors in UTM (Universal Transport Medium) and screened for the presence of RSV. UTM samples negative for RSV were then pooled and spiked with purified RSVA or purified RSVB to mimic patient samples. Total nucleic acid was extracted from each sample as well as positive and negative controls. The RNA from total nucleic acid was converted to cDNA, and the cDNA was used as the template for the amplification of the target NS2 gene as part of a one-step RT-PCR reaction. The primers used for PCR amplification had M13 tags. Subsequently, samples were then purified using a bead-based method and subjected to quantification using TapeStation analysis. Following quantification, samples were diluted and processed with the Applied Biosystems Big Dye Terminator Cycle Sequencing Kit. Finally, samples were sequenced using the Applied Biosystems 3730xl DNA Analyzer. Results were analyzed using Seq Scape 3. All samples were assessed with a series of quality acceptance criteria to determine if the assay was successful and if the resulting sequence was accurate.

Validation

Acceptance Criteria

For sample results to be accepted, they had to meet the specific criteria. The positive control in the run had to depict an electrophoresis band of the size of the expected NS2 amplicon and have ≥ 95% homology to the reference sequence after sequencing. Conversely, the negative controls should not depict an electrophoresis band of the size of the expected NS2 amplicon. Additionally, detection of an electrophoresis band of the size of the expected NS2 amplicon for all samples was necessary. Moreover, every sequenced sample had to exhibit an electropherogram of fluorescence within the range, as confirmed by visual assessment, bi-directional coverage, consensus QV score >20, segment scores >30, and >95% homology with the reference sample. Any sample failing to meet these criteria was deemed unsuccessful. Additionally, any sequence that did not adhere to the specified criteria was deemed inaccurate.

Limit of Detection/Analytical Sensitivity

For each virus, six concentration levels were prepared: for RSVA 3000, 2000, 1000, 800, 600, and 400 copies/mL; and for RSVB 10000, 7500, 5000, 2500, 1000, and 800 copies/mL. Each level was tested in triplicate across three sequencing runs, resulting in a total of nine replicates per dilution level per virus. All samples underwent processing from extraction to sequencing. Limit of detection (LOD) criteria were established: the concentration of the electrophoresis band must be ≥ 4 ng/µL. All positive and negative controls met the acceptance criteria. The LOD was determined to be 600 copies/mL for RSVA and 2500 copies/mL for RSVB.

RSVA Limit of Detection			**RSVB Limit of Detection**		
Copies/mL	Passing Replicates	Accepted LOD	Copies/mL	Passing Replicates	Accepted LOD
3000	9/9	Yes	10000	9/9	Yes
2000	9/9	Yes	7500	9/9	Yes
1000	8/9	Yes	5000	9/9	Yes
800	8/9	Yes	2500	8/9	Yes
600	8/9	Yes	1000	7/9	No
400	6/9	No	800	6/9	No

Intra-assay and Inter-assay Precision/Reproducibility and Accuracy

Precision and accuracy were analyzed using the same sequencing runs and samples[AB11] . For each virus, three concentration levels were prepared: high (1,000,000 copies/mL), medium (100,000 copies/mL), and low (10,000 copies/mL) for both RSVA and RSVB. Each concentration level, along with a negative control, was tested in quintuplicate (5 replicates) across three[KK2] [LB3] runs, resulting in a total of 15 replicates per dilution level and 15 negative replicates per virus. The samples were blinded and run by two different operators.

Precision

Precision criteria were established as follows: all positive spiked samples yield a positive result, negative samples must demonstrate the absence of an electrophoresis band of the size of the expected NS2 amplicon, and the NS2 gene sequence must demonstrate ≥ 95% agreement between replicates. All positive and negative controls met the acceptance criteria. All samples for RSVA and RSVB met the acceptance criteria for precision.

Precision Level	RSVA Precision		RSVB Precision	
	Passing Replicates	*Accepted Precision*	*Passing Replicates*	*Accepted Precision*
High	15/15	Yes	15/15	Yes
Medium	15/15	Yes	15/15	Yes
Low	15/15	Yes	15/15	Yes
Negative	15/15	Yes	15/15	Yes

Accuracy

Accuracy criteria were as follows: all positive spiked samples yield a positive result; negative samples must demonstrate the absence of an electrophoresis band of the size of the expected NS2 amplicon; and bi-directional coverage of the NS2 amplicon must be present in ≥ 90% of all positive samples. Furthermore, all positive and negative controls met the acceptance criteria. All RSVA and RSVB samples met the acceptance criteria for accuracy with a rate of 100%.

Accuracy Level	RSVA Accuracy		RSVB Accuracy	
	Passing Replicates	*Accepted Precision*	*Passing Replicates*	*Accepted Precision*
High	15/15	Yes	15/15	Yes
Medium	15/15	Yes	15/15	Yes
Low	15/15	Yes	15/15	Yes
Negative	15/15	Yes	15/15	Yes

Conclusion

The developed assay yields precise and accurate NS2 gene sequences from nasal swabs infected with RSVA and RSVB. The assay demonstrates equal accuracy and precision in sequencing NS2 gene in both major RSV groups, despite a lower limit of detection in RSVA compared to RSVB. Its robustness is evidenced by consistent and reliable results, even in the face of sample concentration variations. Moreover, the scalability and reproducibility of the assay highlights its utility in both research and clinical settings, particularly for infectious diseases where robust and reliable sequencing methodologies are paramount.

In conclusion, this RSV NS2 gene sequencing assay exemplifies molecular diagnostics and serves as a cornerstone in the ongoing pursuit of targeted antiviral interventions. In the era of personalized medicine, this assay is designed to empower researchers and healthcare professionals in the relentless pursuit of effective RSV management.

References

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