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Rapid Simultaneous Detection of Respiratory Infectious Diseases using Immunoprecipitation and Liquid Chromatography-Tandem Mass Spectrometry

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ABSTRACT

Purpose: To monitor multiple infectious diseases in a fast and sensitive way using immunoprecipitation and selected reaction monitoring.

Methods: Prior to IP, equal amounts of all biotinylated antibodies were pooled together as one antibody panel for this study. The biotinylated antibody panel was added to samples collected via nasopharyngeal swabs in viral transport media (VTM) followed by incubation for 15 minutes at room temperature with rotation at Multimix Tube Rotator. The antigen-antibody complex in VTM was directly subjected to IP using Thermo Scientific™ Pierce™ MS-Compatible IP Kit (Streptavidin). The IP purified samples were then digested for 15 minutes at 70 ºC with vortexing at 1000 rpm using SMART Digest™ Trypsin Kits and analyzed by Thermo Scientific™ Vanquish™ MD HPLC system hyphenated to Thermo Scientific™ TSQ Altis™ MD mass spectrometer. Data processing was performed using Thermo Scientific™ TraceFinder™ LDT software 1.0.

Results: In this study, a total of 12 peptides were successfully monitored (2 to 3 peptides per disease type) simultaneously by LC-MS/MS. The entire sample preparation was finalized to less than 1 hour, reduced from the original starting method of 6 hours. LC-MS run time was also optimized to 5 minutes. The protein precipitation and post sample clean-up were eliminated since IP was sufficient to enrich target protein and purify sample matrix. With criteria of % accuracy ± 20, % RSD < 15, % CV < 15, and R^2 > 0.99, LOQs were determined to be between 0.05 to 1 fmol of the SIL peptides on the LC column.

INTRODUCTION

With recent emergences of new infectious diseases and their variants, there is a need to develop a faster and more accurate analytical tool to detect different respiratory infectious disease viral agents such as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and influenza viruses. Among different viral components, nucleocapsid protein or nucleoprotein (NP) is highly conserved, less post-translational modifications possessed including 0 to 3 potential N-linked glycosylation sites, and mostly specific for each infectious disease virus type.1,2 Therefore, targeting NP is more advantageous to the method development, achieving a much simpler and robust method with minimal subsequent modifications.

This report describes a targeted approach for the simultaneous detection of different respiratory infectious disease viruses using immunoprecipitation (IP) and selected reaction monitoring (SRM). The types of respiratory infectious disease viruses monitored in this report include SARS-CoV-2, influenza A and B viruses, respiratory syncytial virus (RSV), and human coronavirus (HCoV-229E). Multiple viruses were selected to show that this method can distinguish among different disease virus types and can be applied to other infectious disease detection for enveloped viruses with NP components present.

MATERIALS AND METHODS

Sample Preparation

The workflow is described in Figure 1 and more details are provided in the sections below.

Reagent Kits

- Thermo Scientific™ Pierce™ Antibody Biotinylation Kit for IP (Part No. 90407)
- Thermo Scientific™ Pierce™ MS-Compatible Magnetic IP Kit (Streptavidin) (Part No. 90408)
- Thermo Scientific™ SMART Digest™ Trypsin Kit, Soluble (Part No. 60113-101)

Immunoprecipitation: Prior to IP, a bulk amount of antibodies were biotinylated using a Pierce Antibody Biotinylation Kit following the published procedure (Pub. No. MAN0016152). Equal amounts of all biotinylated antibodies were pooled together as one antibody panel for this study. The antibody panel was added to the samples containing each NP stored in VTM after removing the NPSs. The samples were then incubated for 15 minutes at room temperature with a rotation at a fixed speed of 18 rpm using a Multimix Tube Rotator to form the antigen-antibody complex. An aliquot of 100 µL of Pierce Streptavidin Magnetic Beads was dispensed into a 1.5 mL microcentrifuge tube and preconditioned with 500 µL of IP-MS Cell Lysis Buffer. The antigen-antibody complex in VTM was directly added to the preconditioned magnetic beads and incubated for 15 minutes at room temperature with a rotation at a fixed speed of 18 rpm using the Multimix Tube Rotator. The samples were washed three times with 500 µL of IP-MS Wash Buffer A. After removing the third supernatant, 200 µL of IP-MS Wash Buffer B was added for the final wash.

On-beads trypsin digestion: The beads were resuspended with 190 µL of SMART digest buffer and 10 µL of 200 fmol/µL of stable isotope-labeled peptides (SIL peptides, Thermo Scientific™ HeavyPeptide™ AQUA Ultimate) followed by adding 5 µL of trypsin. The samples were then incubated for 15 minutes at 70 ºC with mixing at 1000 rpm. To quench the reaction, 2 µL of 10 % formic acid in water was added. The tube was placed on a magnet and the supernatant was collected in a new 1.5 mL microcentrifuge tube. The collected supernatant was centrifuged for 2 min at a speed of 21,100 x g. The samples were diluted 10 times with 0.1 % formic acid in water prior to LC-MS analysis. To generate the sample matrix for the calibration curve, the beads were resuspended in 200 µL of SMART digest buffer without SIL mixture. After adding 5 µL of trypsin, the remaining steps were followed as described above. Stock solutions of different SIL concertation points were first prepared using serial dilution to avoid dilution of the sample matrix (Table 1). An aliquot of 5 µL of each stock was then added to 95 µL of the sample matrix to make a final concentration of SIL peptides.

Peptide selection: The candidate peptides were selected after checking the protein specificity and similarity from UniProt (www.uniprot.org) and GISAID (www.gisaid.org). The final target peptides were determined based on their LC and SRM performance.

Figure 1. Experimental workflow

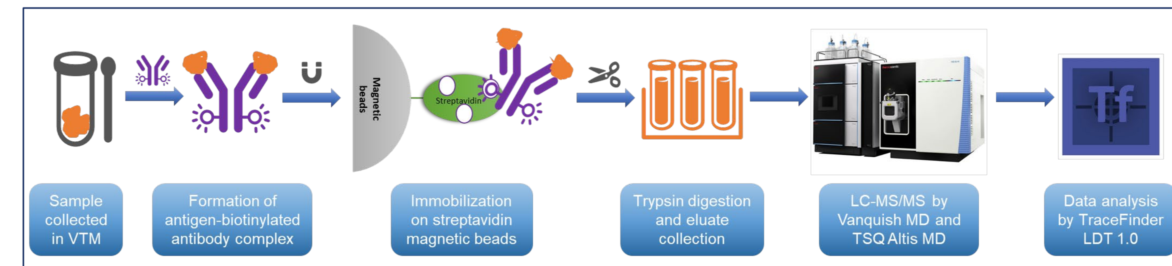


Table 1. Calibration curve generation using SIL peptides

Serial dilution from C11 to C1	Final concentration of SIL peptides (fmol/µL)	Concentration of SIL peptides stock solution (fmol/µL)
C11	10	200
C10	5.0	100
C9	2.5	50
C8	1.0	20
C7	0.50	10
C6	0.25	5
C5	0.10	2
C4	0.050	1
C3	0.025	0.5
C2	0.010	0.2
C1	0.005	0.1

Test Method(s)

LC separation was performed on a Thermo Scientific™ Vanquish™ MD HPLC system using a Thermo Scientific™ Hypersil™ GOLD C18 column (2.1 x 50 mm, 1.9 µm, Part No. 25002052130). Analysis was performed on a Thermo Scientific™ TSQ Altis™ MD mass spectrometer. LC and MS conditions are described in Table 2. Final SRM transitions of the target peptides from each disease are listed in Table 3.

Data Analysis

Data processing was performed using Thermo Scientific™ TraceFinder™ LDT software 1.0. Each data point of the calibration curve was analyzed in triplicate and then fitted with % accuracy ± 20, % RSD < 15, % CV < 15, and R^2 > 0.99 to determine the limits of quantitation (LOQ) for each peptide. The limits of detection (LOD) and linear range were also determined for each peptide.

Table 2. LC and MS conditions

LC gradient			
Time (min)	% A	% B	Curve
0.0	98	2	5
0.5	98	2	5
0.7	90	10	5
3.0	40	60	5
3.3	5	95	5
3.8	5	95	5
3.9	98	2	5
5.0	98	2	5
Separation conditions			
Mobile phase A	0.1 % formic acid in water		
Mobile phase B	0.1 % formic acid in 10: 10: 80 water: isopropanol: acetonitrile (v/v/v)		
Flow rate	0.5 mL/min		
Column temperature	40 ºC		
Injection volume	10 µL		
MS global parameters			
Source type	Heated electrospray ionization (H-ESI)		
Polarity	Positive		
Spray voltage (V)	3500		
Sheath gas (Arb)	50		
Aux gas (Arb)	10		
Sweep gas (Arb)	2		
Ion Transfer tube temp (ºC)	325		
Vaporizer temp (ºC)	350		
Divert Valve A	0.0 min: position 1-6 (waste) 0.5 min: position 1-2 (MS) 3.3 min: position 1-6 (waste)		
SRM scan parameters			
Cycle time (sec)	0.35		
Q1 resolution (FWHM)	0.7		
Q3 resolution (FWHM)	0.7		
CID gas (mTorr)	1.5		
Source fragmentation (V)	0		
Chromatographic peak width (sec)	6		
RF Lens (V)	60		

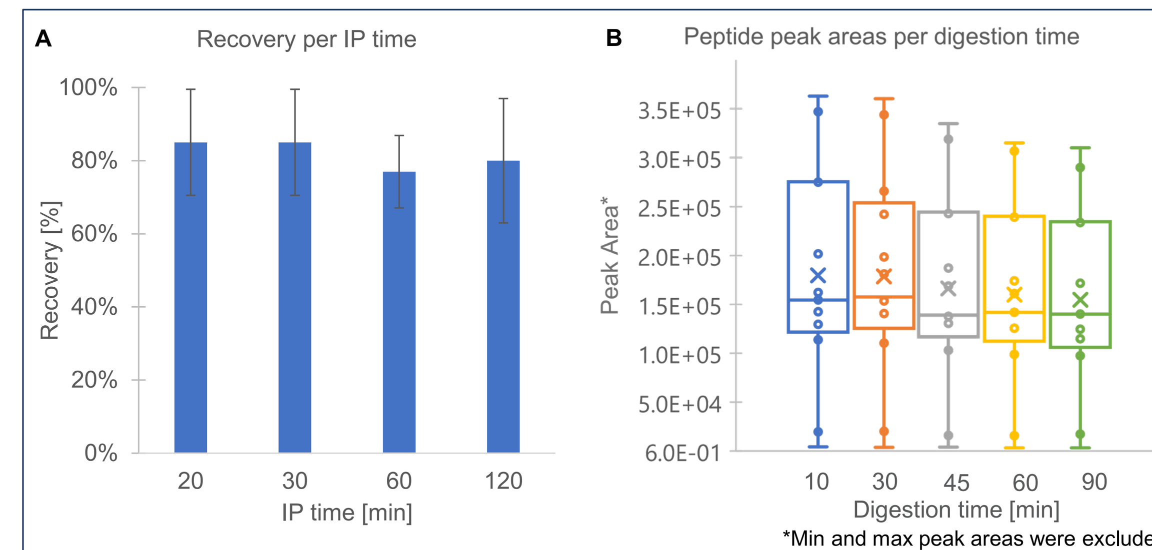
Table 3. List of SRM transitions, retention times, and collision energies (CE) for the endogenous and SIL peptides from each infectious disease

Infectious disease	Peptide sequence	Retention time (min)	Precursor m/z		Product m/z		CE (V)
			Endogenous peptide	SIL peptide	Endogenous peptide	SIL peptide	
SARS-CoV-2	GFYAEGSR	1.60	443.706	448.710	682.32	692.32	16
					519.25	529.26	
					448.22	458.22	
	LNQLESK	1.45	416.232	420.239	604.33	612.34	15
					476.27	484.29	
					363.19	371.20	
	ADETQALPQR	1.52	564.785	569.789	584.35	594.36	20
					513.31	523.32	
					400.23	410.24	
Influenza A	SALILR	1.93	336.723	341.727	585.41	595.42	13
					514.37	524.38	
					401.29	411.30	
	EGYSLVGIDPFK	2.48	662.842	666.849	775.43	783.45	23
					676.37	684.38	
					391.23	399.25	
	GVFELSDEK	2.02	512.253	516.260	720.34	728.36	18
					591.30	599.31	
					478.21	486.23	
Influenza B	TIYFSPIR	2.18	498.779	503.783	782.42	792.43	18
					619.36	629.36	
					472.29	482.30	
	GGGTLVAEAIR	1.97	522.295	527.299	658.39	668.40	19
					559.32	569.33	
					488.28	498.29	
HCoV-229E	AVAAALK	1.54	322.210	326.217	572.38	580.39	13
					473.31	481.32	
					402.27	410.29	
	FLEELNAFTR	2.34	620.322	625.326	721.40	731.41	22
					608.32	618.32	
					423.24	433.24	
RSV	DQLLSSSK	1.62	439.234	443.242	634.38	42.39	16
					521.29	529.31	
					408.21	416.22	
	NQDLYDAAK	1.62	519.248	523.255	795.39	803.40	19
					680.36	688.38	
					567.28	575.29	

RESULTS

The workflow was optimized from sample preparation to LC-MS analysis. The protein precipitation and post sample clean-up were eliminated since IP was sufficient to enrich target protein and purify the sample matrix. From the IP procedure, the entire incubation steps for antigen-antibody complex formation and immobilization on the magnetic beads were reduced to 30 minutes (originally 2 hours). Figure 2A shows a comparable recovery when using a different IP time. Additionally, the amount of beads used was adjusted to 100 µL for sufficient binding capacity of the pooled antibody panel used in this report.

Figure 2. Workflow optimization



The digestion step was also optimized. As shown in Figure 2B, the measured peak areas of target peptides were comparable across different digestion incubation times from 10 to 90 minutes. This data supports that a shorter digestion time can generate an almost identical sample digest as a longer digestion time (90 min). To accommodate practical hands-on time, 5 minutes were added so a final method was set to 15 minutes of digestion incubation time. The reduction of trypsin digestion time was achieved due to a generation of a much cleaner sample matrix by IP. The entire sample preparation was finalized to less than 1 hour, reduced from the original starting method of 6 hours. LC-MS run time was also optimized to 5 minutes.

In this study, a total of 12 peptides were successfully monitored (2 to 3 peptides per disease type) simultaneously by LC-MS/MS. Table 4 lists LOD, LOQ, linear dynamic range, and R^2 values for each SIL peptide. Great linearity was observed for all the peptides with R^2 values higher than 0.99 as shown in Table 4 and Figure 3. Also, three additional graphs ranging from 0 to 2.5 fmol are included in Figure 3, supporting a reproducible measurement at lower concentration points. With criteria of % accuracy ± 20, % RSD < 15, % CV < 15, and R^2 > 0.99, LOQs were determined to be between 0.05 to 1 fmol of the SIL peptides on the LC column.

The representative retention times of all the target peptides are shown in Figure 4A, starting from 1.45 min to 2.48 min of an observed peak apex. The variation of detected retention time was determined to be less than ± 0.01 minutes over the analyses of the calibration curve as shown in Figure 4B. Therefore, a fast 5-minute LC gradient achieved reliable detection of the target peptides.

Table 4. Determined analytical properties of the method from the calibration curve including LODs, LOQs, linear range, and R^2 values

	Peptide Sequence	LOD (fmol on column)	LOQ (fmol on column)	Linear Range (fmol on column)	R^2
SARS-CoV-2	GFYAEGSR	0.10	0.25	0.25 - 100	0.9971
	LNQLESK	0.05	0.05	0.05 - 100	0.9992
	ADETQALPQR	0.25	0.25	0.25 - 100	0.9979
Influenza A	SALILR	0.50	0.50	0.50 - 100	0.9988
	EGYSLVGIDPFK	0.10	0.25	0.25 - 100	0.9901
	GVFELSDEK	0.25	0.25	0.25 - 100	0.9970
Influenza B	TIYFSPIR	0.10	0.25	0.25 - 100	0.9985
	GGGTLVAEAIR	0.10	0.10	0.10 - 100	0.9990
HCoV-229E	AVAAALK	1.00	1.00	1.00 - 100	0.9974
	FLEELNAFTR	0.25	0.50	0.50 - 100	0.9951
RSV	DQLLSSSK	0.50	0.50	0.50 - 100	0.9903
	NQDLYDAAK	1.00	1.00	1.00 - 100	0.9939

Figure 3. Calibration curves of all the target peptides over their corresponding linear ranges. Three calibration curves of the peptides LNQLESK, SALILR, and TIYFSPIR at low calibration points from 0 to 2.5 fmol.

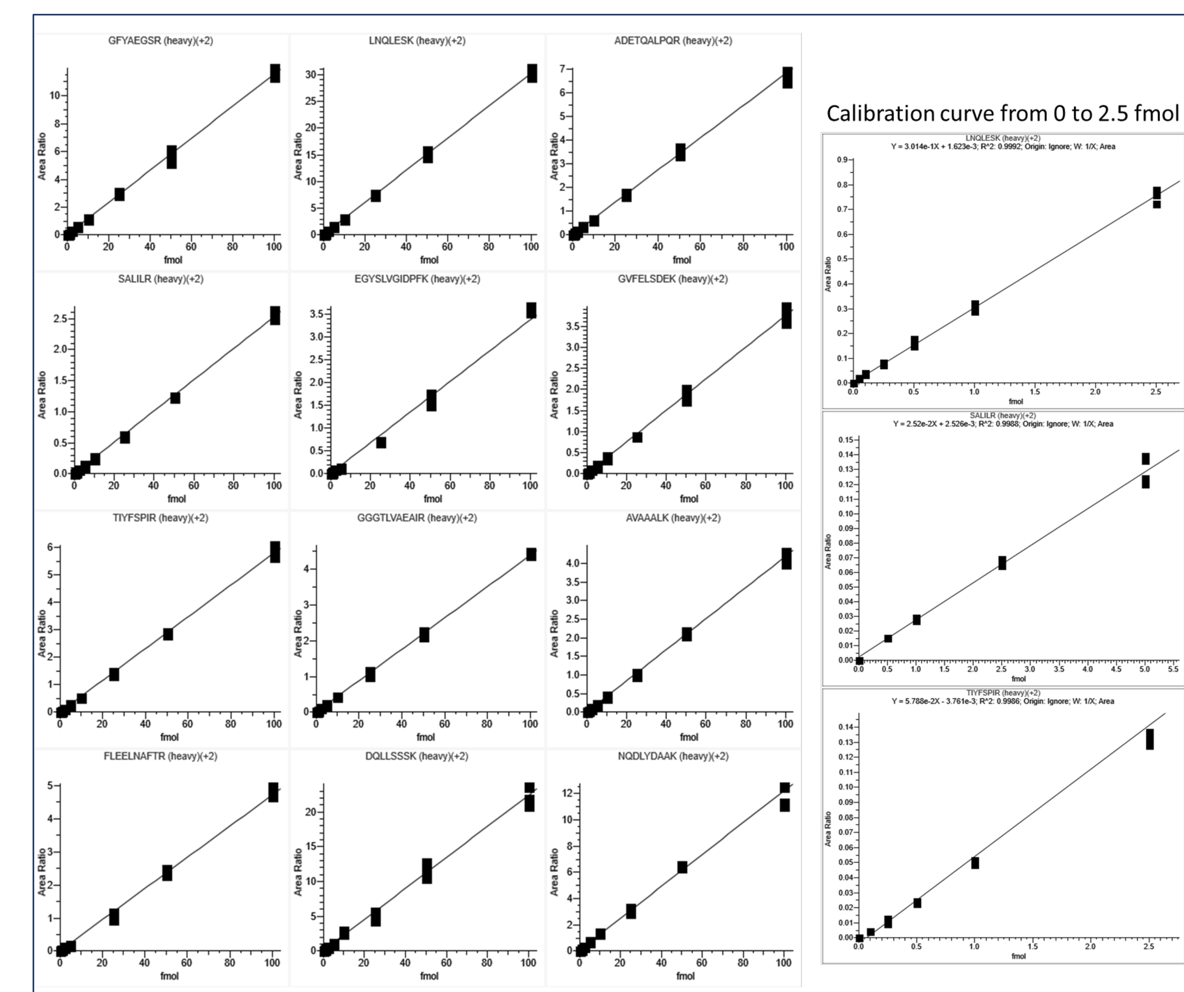
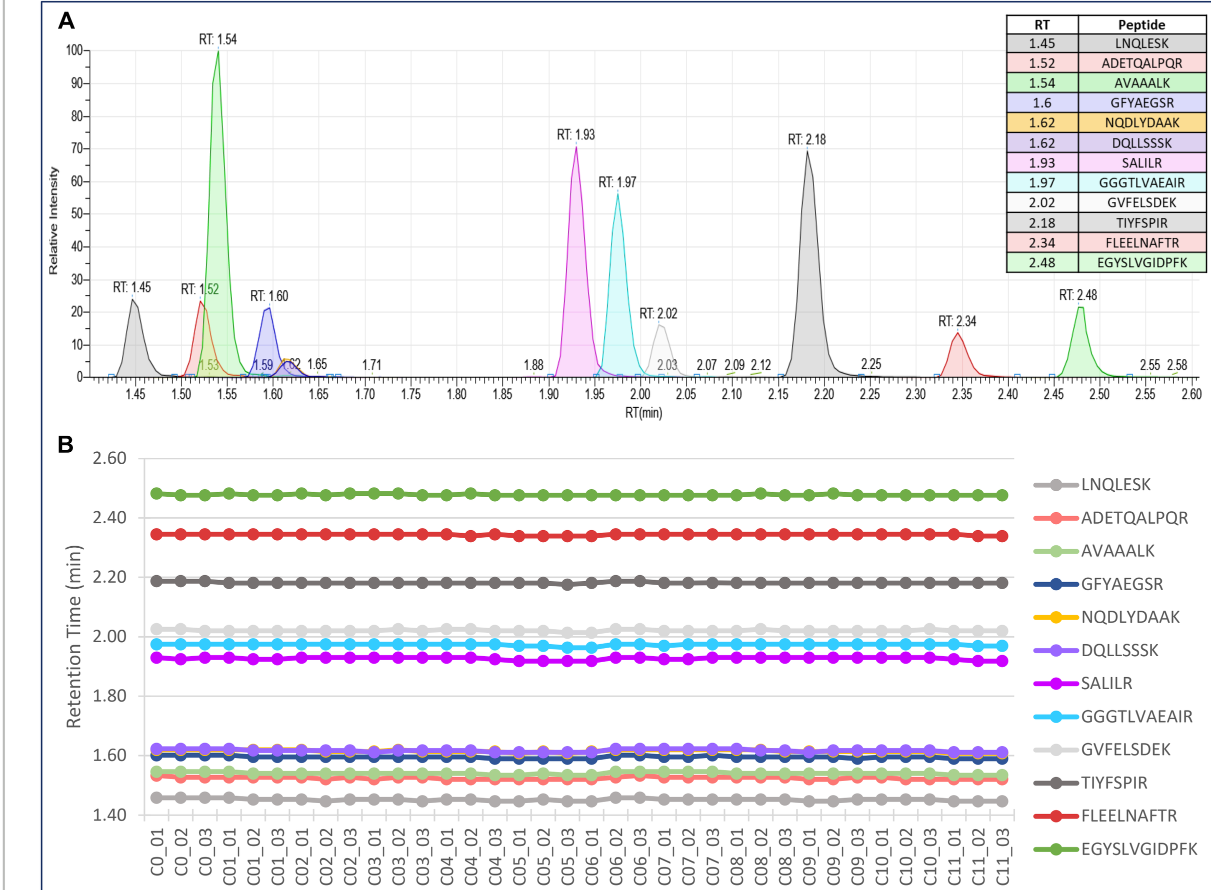


Figure 4. Representative retention times of all the target peptides (A) and variation of detected retention time (B)



CONCLUSIONS

- We have shown successful implementation and optimization of IP and SRM methods to simultaneously monitor four types of infectious diseases by targeting the NP component of enveloped viruses.
- The workflow was optimized to less than 1-hour sample preparation and 5-minute LC-MS analysis.
- The IP method generated a clean and MS-compatible sample matrix, providing reliable quantification of 0.05 to 1 fmol of the peptides on the column.
- This optimized and fast process increases sample throughput and ultimately expedites turn-around time.
- Incorporation of the Thermo Scientific™ KingFisher™ sample purification system can reduce 70% of the manual steps, increase consistency, and facilitate greater sample throughput for high-volume laboratories.

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