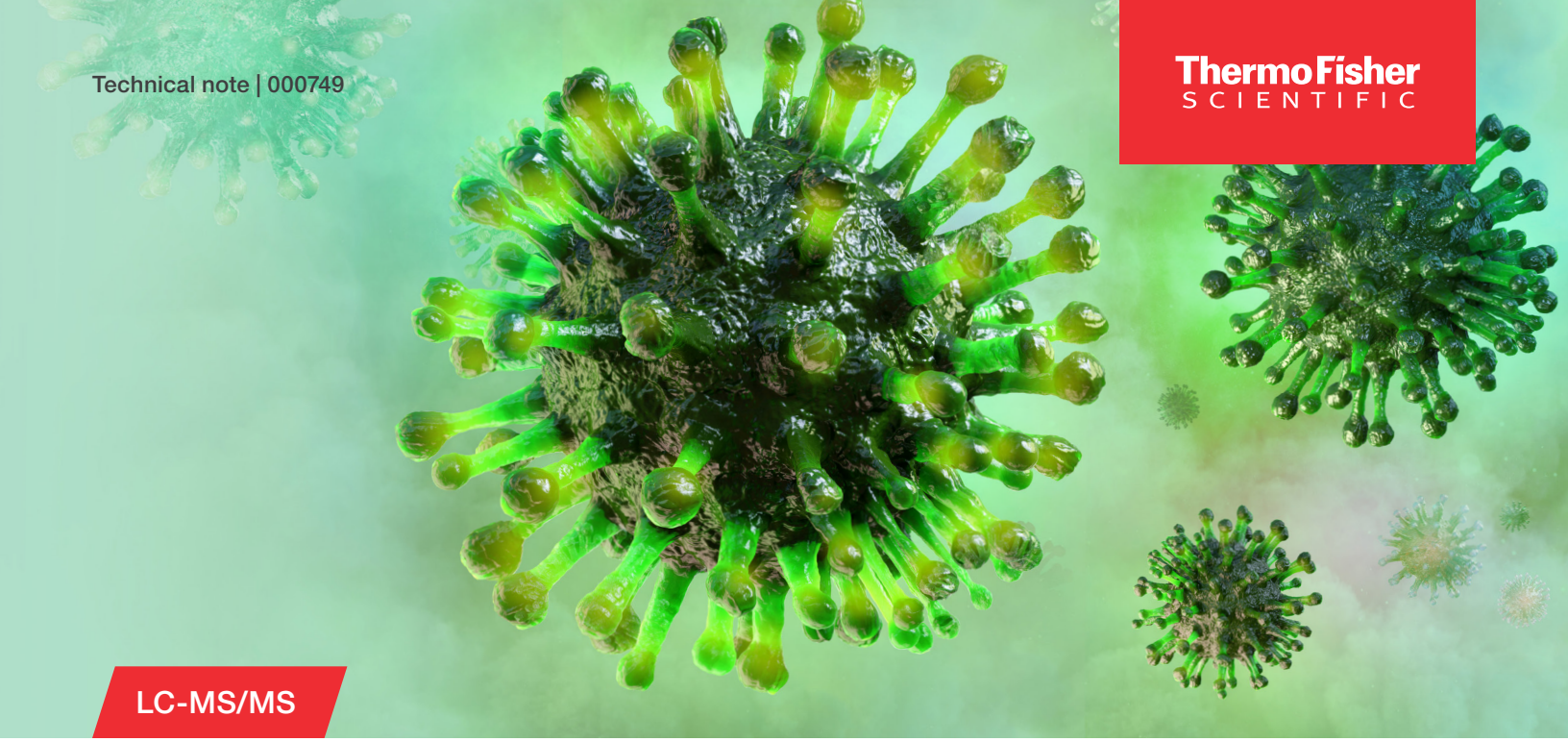
Technical note | 000749



LC-MS/MS

Simultaneous detection of respiratory infectious diseases using immunoprecipitation and LC-MS/MS

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Keywords

TSQ Altis MD MS, Vanquish MD HPLC, TraceFinder LDT software, Hypersil GOLD C18 column, SMART Digest Trypsin Kit, immunoprecipitation, magnetic beads, streptavidin, SARS-CoV-2, COVID-19, influenza virus, respiratory syncytial virus, RSV, HCoV-229E, peptide quantitation, nucleocapsid protein, nucleoprotein, LC-MS, mass spectrometry

Application benefits

- Simple and robust approach by targeting the nucleoprotein component of the enveloped virus
- Quick sample preparation taking < 1 hour
- MS-compatible and clean sample matrix generation by immunoprecipitation
- Highly targeted, sensitive, and confident detection by immunoprecipitation and selected reaction monitoring

Goal

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

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. NP has fewer post-translational modifications, which include 0 to 3 potential N-linked glycosylation sites, and most of those modifications are specific to a particular disease 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.

Experimental

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

Reagent kits

- Thermo Scientific™ Pierce™ Antibody Biotinylation Kit for IP (P/N 90407)
- Thermo Scientific™ Pierce™ MS-Compatible Magnetic IP Kit (streptavidin) (P/N 90408)
- Thermo Scientific™ SMART Digest™ Trypsin Kit, soluble (P/N 60113-101)

Viral transport media preparation

The viral transport media (VTM) was prepared in accordance with CDC guidelines.[3] An aliquot of 10 mL of inactivated fetal bovine serum (Gibco) was added to a 500 mL bottle of Hanks Buffered Salt Solution (MP Biomedicals). Gentamicin (Gibco) and amphotericin B (Gibco) were added to make final concentrations of 100 and 0.5 µg/mL, respectively. The final VTM was aliquoted to 1 mL and stored at 4 °C.

Sample preparation in nasal fluids

The NPs and antibodies used in this report are listed in Table 1. An aliquot of 5 µL of each NP (concentration of 1 mg/mL) was spiked into 50 µL of pooled nasal fluids from healthy donors (Innovative Research). Samples were vortexed to mix and pipetted onto nasopharyngeal swabs. The nasopharyngeal swabs were then stored in 1 mL of VTM at 25 °C for 10 minutes followed by vortexing for 30 seconds.

Immunoprecipitation

Prior to IP, a bulk amount of antibodies were biotinylated using a Pierce Antibody Biotinylation Kit following the published procedure (Pub. No. MAN0016152). The biotinylated antibodies were aliquoted and stored at -20 °C. A fresh aliquot was used right before IP. 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 nasopharyngeal swabs. 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 (VWR) 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. The magnetic beads were preconditioned with 500 µL of IP-MS Cell Lysis Buffer. To mix the beads, gentle pipetting multiple times is recommended throughout the IP process. Vortexing is not recommended as it is too disruptive to the bead mixture. 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 sample tube was placed on a magnet and the supernatant was

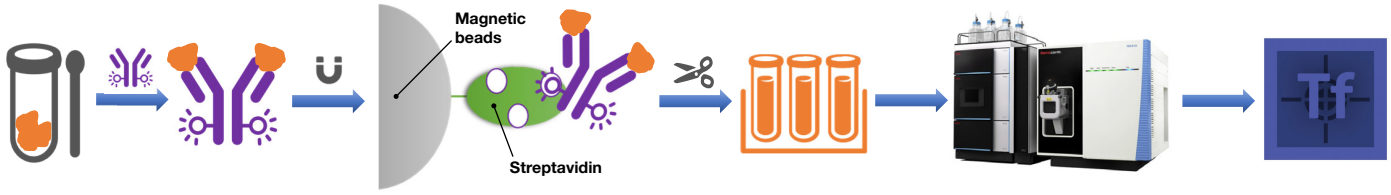

Figure 1. Experimental workflow

Table 1. Information of nucleoproteins and nucleoprotein antibodies of different respiratory infectious diseases

Target respiratory infectious disease	Nucleoprotein (vendor)	Antibody (vendor)
SARS-CoV-2	• SARS-CoV-2 nucleocapsid protein (Invitrogen)	• SARS-CoV-2 nucleocapsid anti-virus clone (Invitrogen)
Influenza virus A	Pool of: • Influenza A H1N1 (A/Wisconsin/588/2019) / (A/Victoria/2570/2019) nucleoprotein (Sino Biological) • Influenza A H3N2 (A/Cambodia/e0826360/2020) nucleoprotein (Sino Biological)	• Pan influenza A nucleoprotein antibody (Sino Biological)
Influenza virus B	• Influenza B (B/Washington/02/2019) nucleoprotein (Sino Biological)	• Pan influenza B nucleoprotein antibody (Sino Biological)
HCoV-229E	• HCoV-229E nucleocapsid protein (Sino Biological)	• HCoV-229E nucleocapsid antibody (Sino Biological)
RSV	• RSV nucleoprotein (Creative Diagnostics)	• RSV nucleoprotein anti-virus clone (Bioss)

removed. An aliquot of 500 µL of IP-MS Wash Buffer A was added and mixed by gentle pipetting multiple times. This wash step was repeated three times. After adding the third IP-MS Wash Buffer A, the samples were transferred to a new 1.5 mL microcentrifuge tube to remove residual surfactant from the IP-MS Cell Lysis Buffer left on the tube wall. After removing the third supernatant, 200 µL of IP-MS Wash Buffer B was added for the final wash. The tube was placed on a magnet and the supernatant was removed.

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 °C with mixing at 1,000 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 × 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 2). 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.

Table 2. Calibration curve generation using SIL peptides

Serial dilution from C11 to C1	Final concentration of SIL peptides (fmol/mL)	Concentration of SIL peptides stock solution (fmol/mL)
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

Liquid chromatography

LC separation was performed on a Thermo Scientific™ Vanquish™ MD HPLC system using a Thermo Scientific™ Hypersil GOLD™ C18 column (2.1 × 50 mm, 1.9 µm, P/N 25002-052130). The LC gradient and other separation conditions are described in Table 3.

Mass spectrometry

Analysis was performed on a Thermo Scientific™ TSQ Altis™ MD mass spectrometer with settings described in Table 4. 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. Final SRM transitions of the target peptides from each disease are listed in Table 5.

Data processing

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 3. LC gradient and separation 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 (mL/min)	0.5		
Column temperature (°C)	40		
Injection volume (µL)	10		

Table 4. MS global and SRM scan parameters

Global parameters	
Source type	Heated electrospray ionization (H-ESI)
Polarity	Positive
Spray voltage (V)	3,500
Sheath gas (Arb)	50
Aux gas (Arb)	10
Sweep gas (Arb)	2
Ion transfer tube temperature (°C)	325
Vaporizer temperature (°C)	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 (s)	0.35
Q1 resolution (FWHM)	0.7
Q3 resolution (FWHM)	0.7
CID gas (mTorr)	1.5
Source fragmentation (V)	0
Chromatographic peak width (s)	6
RF Lens (V)	60

Table 5. list of SRM transitions

Infectious disease	Peptide sequence	Retention time (min)	Precursor *m/z*		Product *m/z*		CE (V)
			Endogenous peptide	SIS peptide	Endogenous peptide	SIS 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 and discussion

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 the 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.

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 the 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. The 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 6 lists LOD, LOQ, linear dynamic range, and R^2 values for each SIL peptide. Great linearity was observed for all peptides, with R^2 values higher than 0.99 as shown in Table 6 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.

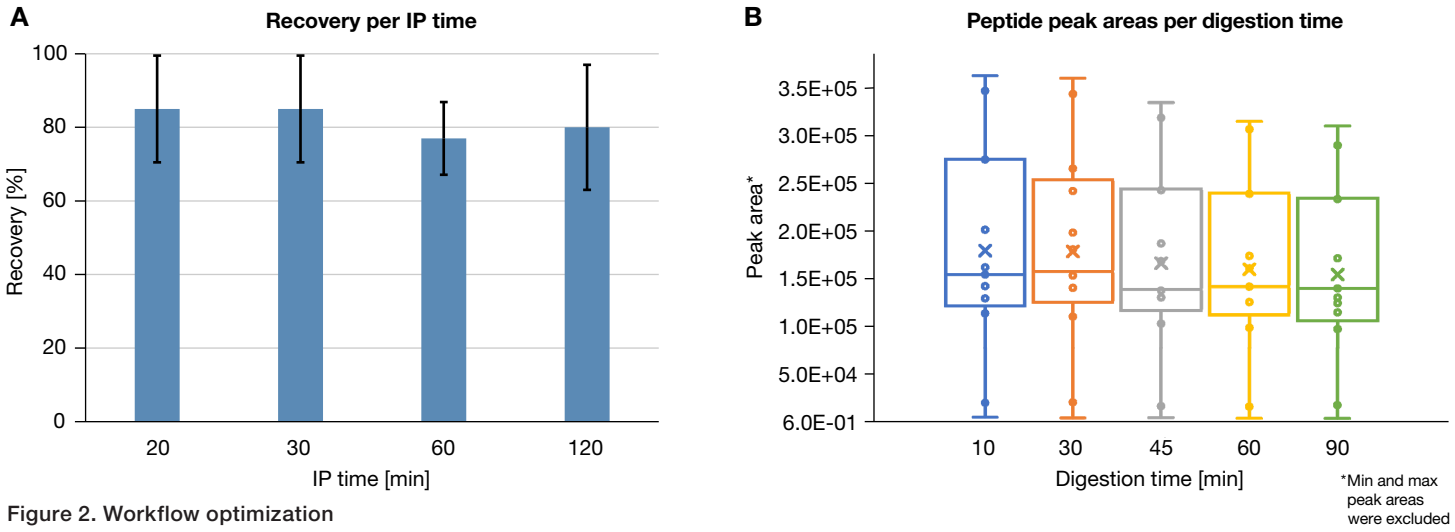

Figure 2. Workflow optimization

Table 6. 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)	Type	Weighting	Origin	R^2
SARS-CoV-2	GFYAEGSR	0.10	0.25	0.25–100	Linear	1/x	Ignore	0.9971
	LNQLESK	0.05	0.05	0.05–100	Linear	1/x	Ignore	0.9992
	ADETQALPQR	0.25	0.25	0.25–100	Linear	1/x	Ignore	0.9979
Influenza A	SALILR	0.50	0.50	0.50–100	Linear	1/x	Ignore	0.9988
	EGYSLVGIDPFK	0.10	0.25	0.25–100	Linear	1/x	Ignore	0.9901
	GVFELSDEK	0.25	0.25	0.25–100	Linear	1/x	Ignore	0.9970
Influenza B	TIYFSPIR	0.10	0.25	0.25–100	Linear	1/x	Ignore	0.9985
	GGGTLVAEAIR	0.10	0.10	0.10–100	Linear	1/x	Ignore	0.9990
HCoV-229E	AVAAALK	1.00	1.00	1.00–100	Linear	1/x	Ignore	0.9974
	FLEELNAFTR	0.25	0.50	0.50–100	Linear	1/x	Ignore	0.9951
RSV	DQLLSSSK	0.50	0.50	0.50–100	Linear	1/x	Ignore	0.9903
	NQDLYDAAK	1.00	1.00	1.00–100	Linear	1/x	Ignore	0.9939

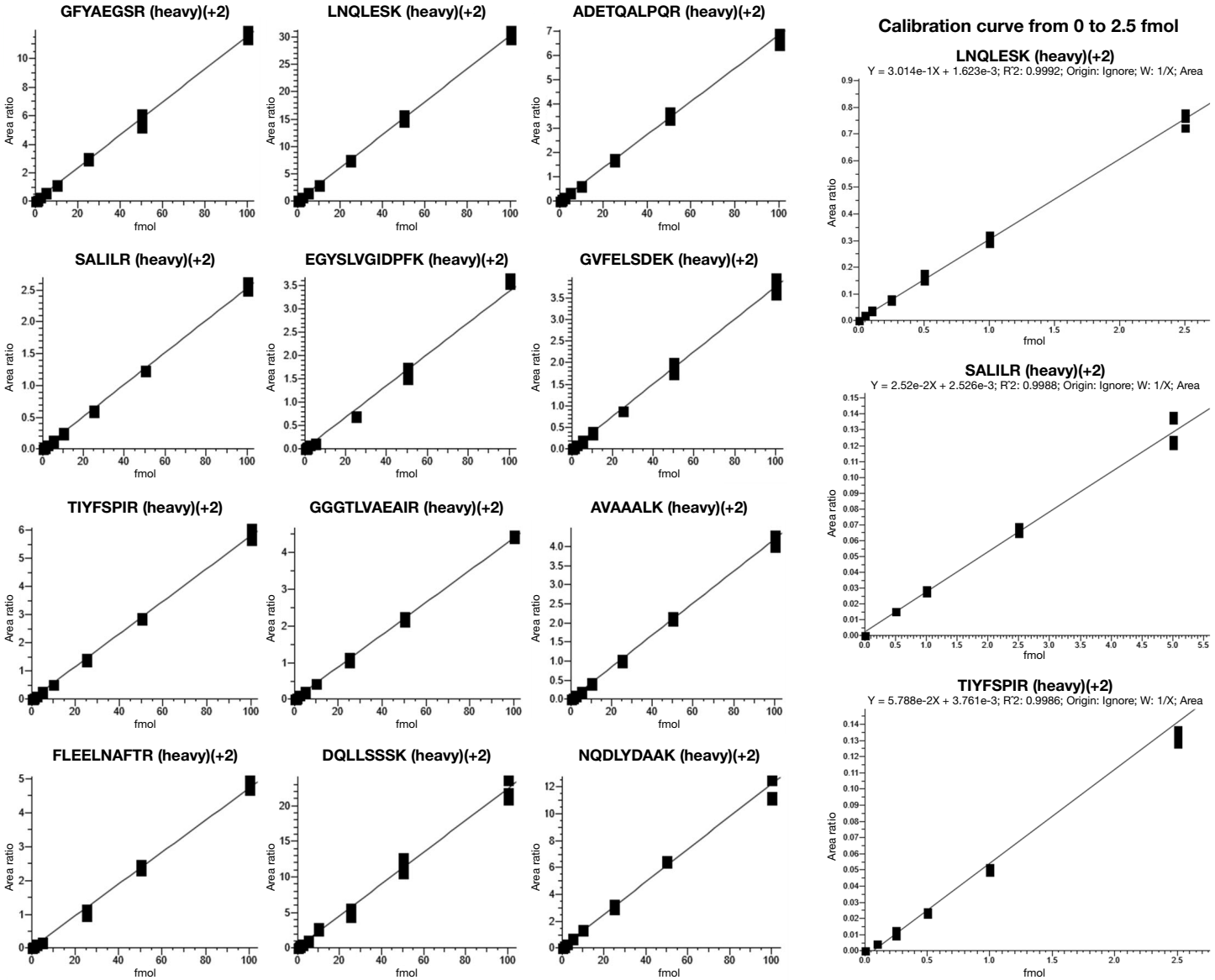

Figure 3. Calibration curves of all the target peptides over their corresponding linear ranges. Three calibration curves of the peptides LNQLESK, SALILR, and TIYFSPIR at low calibration points from 0 to 2.5 fmol.

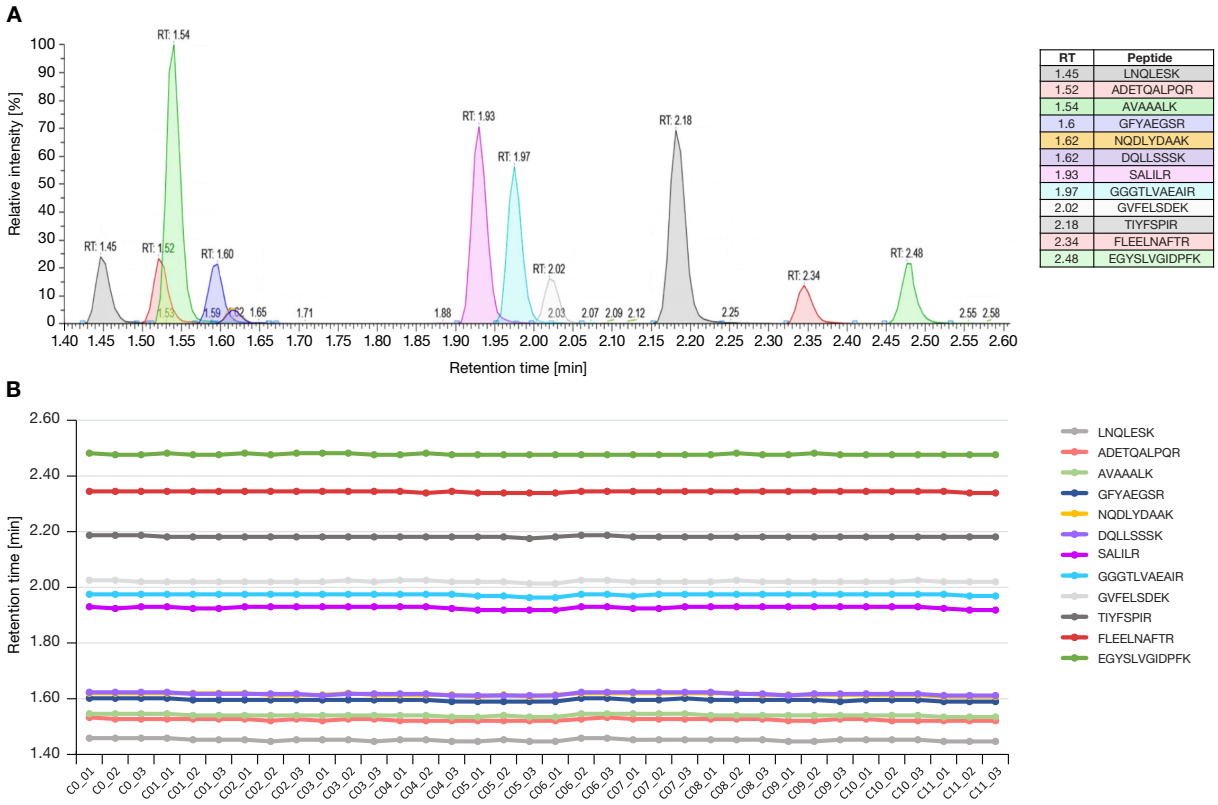

Figure 4. Representative retention times of all the target peptides (A) and variation of detected retention time (B)

Conclusion

In this report, 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 of sample preparation and a 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.

Previous technical notes on a robust detection of SARS-CoV-2 in different sample collections and using peptide enrichment techniques are available online (TN000055 and TN000526).

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