SignalScan™ targeted LCMS/MS assay to profile changes in protein and phosphorylation levels with SARS-CoV-2 infection and treatment

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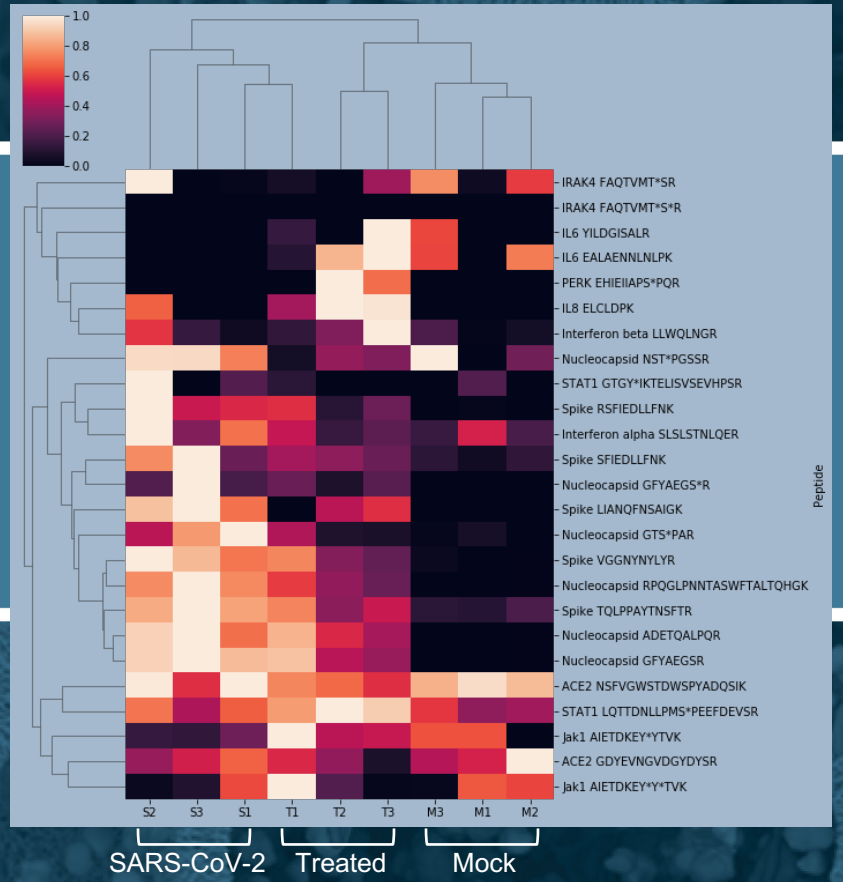

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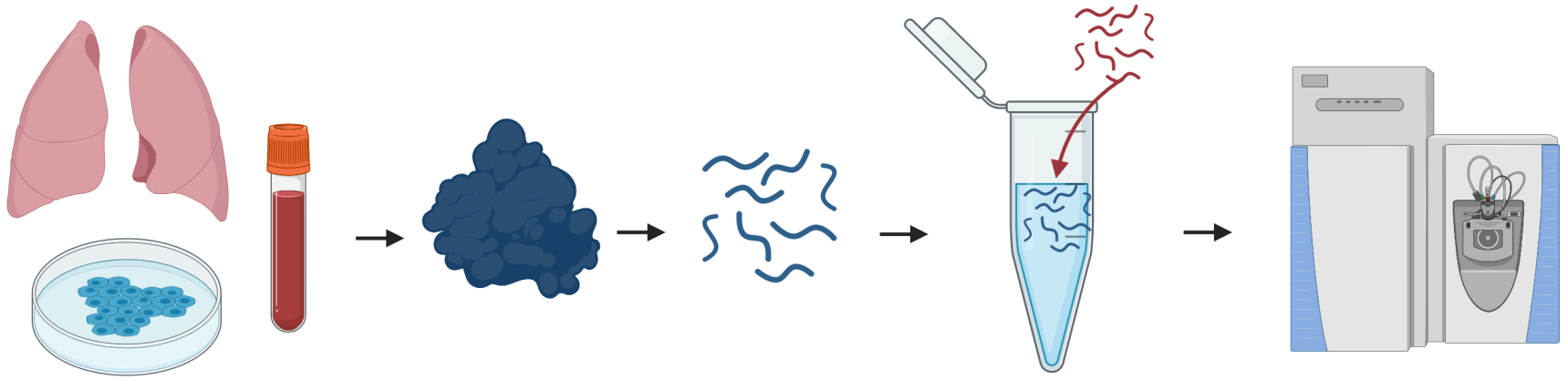
Abstract

Infection with the novel coronavirus SARS-CoV-2 has resulted in a worldwide viral pandemic. Here we present a targeted LCMS/MS-based assay to measure changes in cell signaling with viral infection and treatment. Assay targets represent viral and host proteins, including the SARS-CoV-2 Spike and Nucleocapsid proteins and human proteins such as ACE2, cytokines, and intracellular signaling markers, which are involved in viral entry, the innate immune response, interferon signaling, translational activity, and viral RNA detection. Changes in total protein levels, sites of phosphorylation, and cleavage events are quantified.

We synthesized and quantified a mixture of over two dozen heavy labeled (13C, 15N) Stable Isotope Standard (SIS) peptides and phosphopeptides representing both viral and host proteins. In the SignalScan workflow, one pmol of each SIS peptide was spiked into one microgram of tryptic peptides derived from a human or primate cell line, LCMS/MS was performed on a Fusion Lumos mass spectrometer using the targeted SureQuant SIS-Assisted Scanning (SISAS) method, and data analysis was performed in Skyline.

Cells were mock-infected or infected with SARS-CoV-2 and treated or untreated with a kinase inhibitor prior to lysis and digestion. Quantitative profiling of viral peptides and phosphopeptides revealed decreases in viral protein phosphorylation upon kinase inhibitor treatment. Host protein expression and phosphorylation changes were also measured. The SISAS method for targeted analysis resulted in improved coverage of assay targets over data-dependent analysis.

Utilizing the SignalScan workflow enables deeper, more consistent profiling of target peptides for accurate quantification of both protein levels and post-translational modifications in unenriched samples, greatly simplifying both sample preparation and reducing starting sample requirements. This simplified protocol enables researchers to easily assay critical viral and host signaling events.



SignalScan™ Peptide Mix (SARS-CoV-2)

Protein	PTM	Peptide
Spike		TQLPPAYTNSFT**R**
Spike		VGGNYNYLY**R**
Spike	R815 uncleaved	RSFIEDLLFN**K**
Spike		SFIEDLLFN**K**
Spike		LIANQFNSAIG**K**
Nucleocapsid		RPQGLPNNTASWFTALTQHG**K**
Nucleocapsid		ADETQALPQ**R**
Nucleocapsid		GFYAEGS**R**
Nucleocapsid	pS176	GFYAEGS*__R__
Nucleocapsid	pT198	NST*PGSS__R__
Nucleocapsid	pS206	GTS*PA__R__
ACE2		GDYEVNGVDGYDYS**R**
ACE2		NSFVGWSTDWSPYADQSI**K**
IL6		EALAENNLNLP**K**
IL6		YILDGISAL**R**
IL8		ELCLDP**K**
Interferon alpha		SLSLSTNLQE**R**
Interferon beta		LLWQLNG**R**
Jak1	pY1034	AIETDKEY*YTV**K**
Jak1	pY1034/pY1035	AIETDKEY*Y*TV**K**
STAT1	pS727	LQTTDNLLPMS*PEEFDEVS**R**
STAT1	pY701	GTGY*IKTELISVSEVHPS**R**
IRAK4	pT345	FAQTVMT*S**R**
IRAK4	pT345/pS346	FAQTVMT*S*__R__
PERK	pS715	EHIEIIAPS*PQ**R**

Nucleocapsid protein

MSDNGPQNQRNAPRITFGGPSDSTGSNQNGERSGARSKQRRPQGLPNNTA
SWFTALTQHGKEDLKFPRGQGVPINTNSSPDDQIGYYRRATRRIRGGDGK
MKDLSPRWYFYYLGTGPEAGLPYGANKDGIIWVATEGALNTPKDHIGTRN
PANNAAIVLQLPQGTTLPKGFYAEGSRGGSQASSRSSSRSRNSSRNSTPG
SSRGTSPARMAGNGGDAALALLLLDRLNQLESKMSGKGQQQQGQTVTKKS
AAEASKKPRQKRTATKAYNVTQAFGRRGPEQTQGNFGDQELIRQGTDYKH
WPQIAQFAPSASAFFGMSRIGMEVTPSGTWLTYTGAIKLDDKDPNFKDQV
ILLNKHIDAYKTFPPTEPKKDKKKKADETQALPQRQKKQQTVTLLPAADL
DDFSKQLQQSMSSADSTQA

RNA-binding domain
Dimerization domain

Left: Peptides included in SARS-CoV-2 SignalScan™ Peptide Mix. Peptides in blue are derived from SARS-CoV-2 proteins. Peptides in black represent human proteins. Proteins and phosphorylation sites included in these peptides were selected to enable researchers to monitor both viral infection and host response. All peptides are stable-isotope labeled on their carboxy-terminal amino acid ($^{15}N_4{}^{13}C_6$-Arg or $^{15}N_2{}^{13}C_6$-Lys), designated by bold **R** or **K**. Phosphorylated residues are designated with *.

Above and Right: Sequences of SARS-CoV-2 Nucleocapsid and Spike proteins. Underlined sequences indicate peptides included in SignalScan Peptide Mix.

Spike protein

MFVFLVLLPLVSSQCVNLTTRTQLPPAYTNSFTRGVYYPDKVFRSSVLHS
TQDLFLPFFSNVTWFHAIHVSGTNGTKRFDNPVLPFNDGVYFASTEKSNI
IRGWIFGTTLDSKTQSLLIVNNATNVVIKVCEFQFCNDPFLGVYYHKNNK
SWMESEFRVYSSANNCTFEYVSQPFLMDLEGKQGNFKNLREFVFKNIDGY
FKIYSKHTPINLVRDLPQGFSALEPLVDLPIGINITRFQTLLALHRSYLT
PGDSSSGWTAGAAAYYVGYLQPRTFLLKYNENGTITDAVDCALDPLSETK
CTLKSFTVEKGIYQTSNFRVQPTESIVRFPNITNLCPFGEVFNATRFASV
YAWNRKRISNCVADYSVLYNSASFSTFKCYGVSPTKLNDLCFTNVYADSF
VIRGDEVRQIAPGQTGKIADYNYKLPDDFTGCVIAWNSNNLDSKVGGNYN
YLYRLFRKSNLKPFERDISTEIYQAGSTPCNGVEGFNCYFPLQSYGFQPT
NGVGYQPYRVVVLSFELLHAPATVCGPKKSTNLVKNKCVNFNFNGLTGTG
VLTESNKKFLPFQQFGRDIADTTDAVRDPQTLEILDITPCSFGGVSVITP
GTNTSNQVAVLYQDVNCTEVPVAIHADQLTPTWRVYSTGSNVFQTRAGCL
IGAEHVNNSYECDIPIGAGICASYQTQTNSPRRARSVASQSIIAYTMSLG
AENSVAYSNNSIAIPTNFTISVTTEILPVSMTKTSVDCTMYICGDSTECS
NLLLQYGSFCTQLNRALTGIAVEQDKNTQEVFAQVKQIYKTPPIKDFGGF
NFSQILPDPSKPSKRSFIEDLLFNKVTLADAGFIKQYGDCLGDIAARDLI
CAQKFNGLTVLPPLLTDEMIAQYTSALLAGTITSGWTFGAGAALQIPFAM
QMAYRFNGIGVTQNVLYENQKLIANQFNSAIGKIQDSLSSTASALGKLQD
VVNQNAQALNTLVKQLSSNFGAISSVLNDILSRLDKVEAEVQIDRLITGR
LQSLQTYVTQQLIRAAEIRASANLAATKMSECVLGQSKRVDFCGKGYHLM
SFPQSAPHGVVFLHVTYVPAQEKNFTTAPAICHDGKAHFPREGVFVSNGT
HWFVTQRNFYEPQIITTDNTFVSGNCDVVIGIVNNTVYDPLQPELDSFKE
ELDKYFKNHTSPDVDLGDISGINASVVNIQKEIDRLNEVAKNLNESLIDL
QELGKYEQYIKWPWYIWLGFIAGLIAIVMVTIMLCCMTSCCSCLKGCCSC
GSCCKFDEDDSEPVLKGVKLHYT

S1 Domain
Receptor Binding Domain
S2 Domain
| = cleavage site

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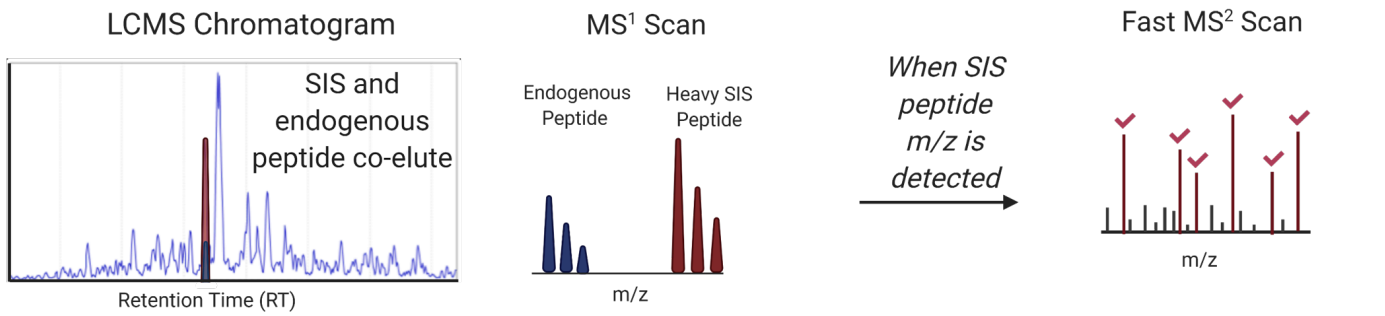
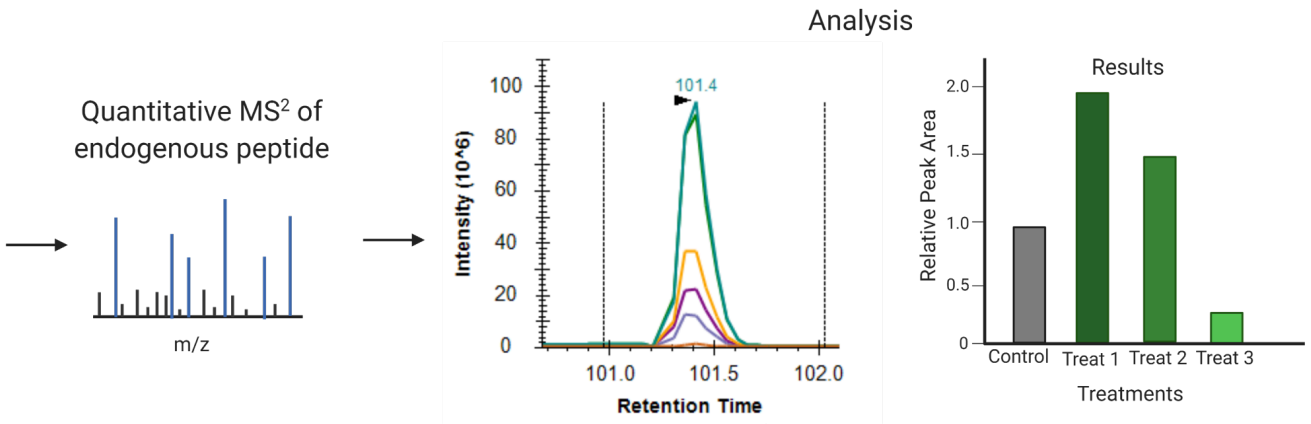
Stable Isotope Standard Assisted Scanning (SISAS) method



MS¹ scans only until stable isotope standard (SIS) peptide begins eluting; no RT scheduling required

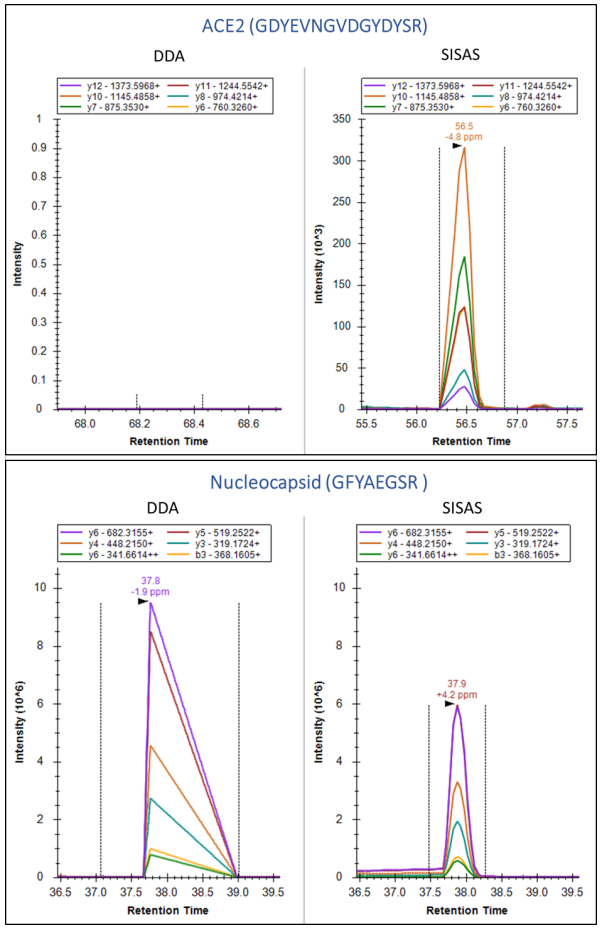
Matching fragments of SIS peptide confirm identity



Integrate peak areas from endogenous peptide MS² fragments

Compare relative changes across treatment groups

DDA vs. SISAS

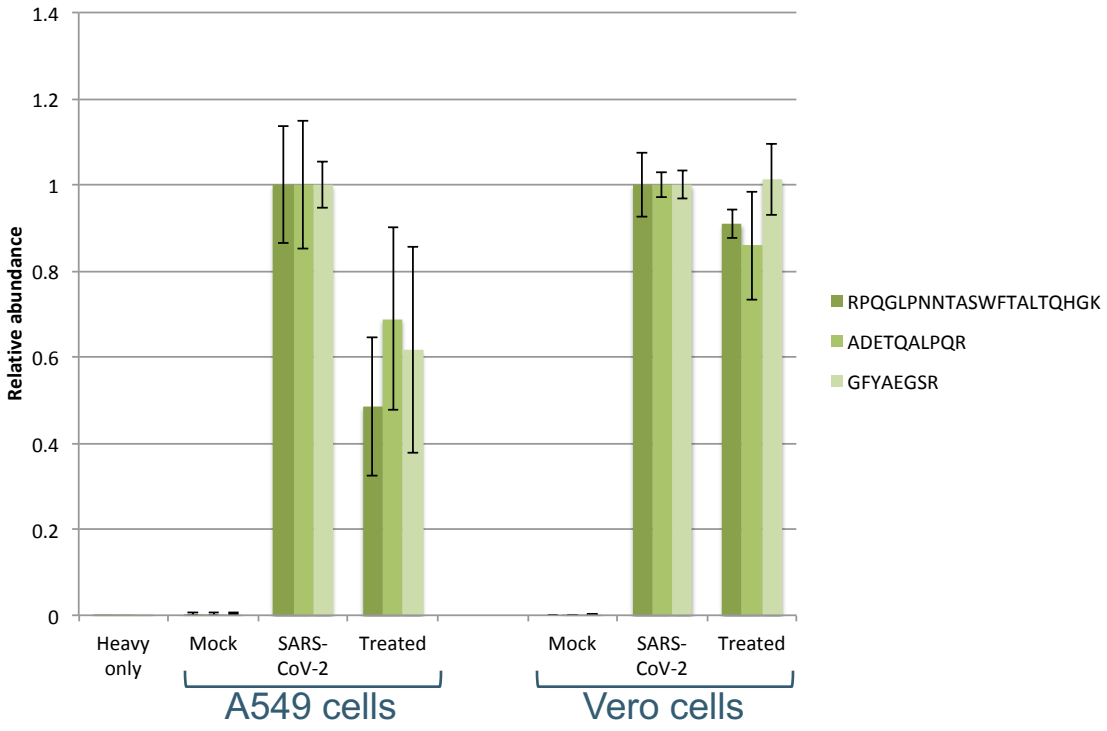


Product ion chromatograms, visualized in Skyline, of peptides from the human ACE2 protein (top) and the SARS-CoV-2 Nucleocapsid protein (bottom), derived from virally-infected A549 cells. Using a standard data-dependent analysis (DDA) LCMS/MS method (left panels) results in no signal or poor peak shape due to instrument duty cycle. A Stable Isotope Standard Assisted Scanning (SISAS) method (right panels) results in consistently detected peptides with quality peak shapes resulting in confident peptide and protein quantitaton.

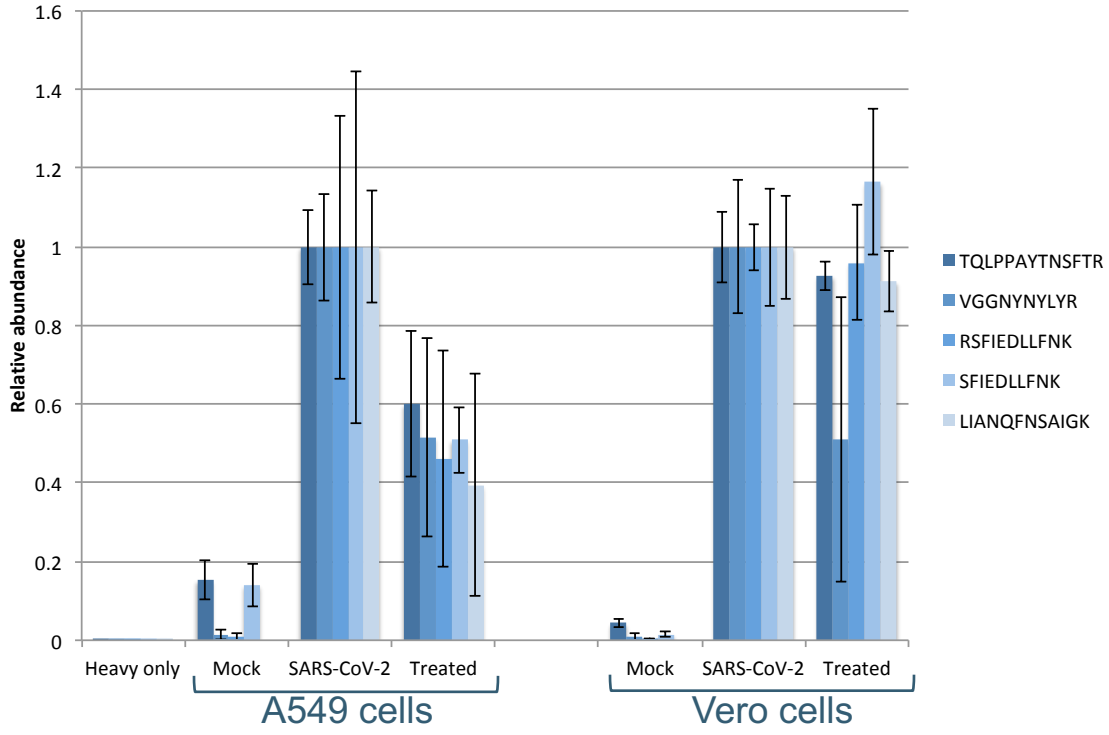
Stable Isotope Standard Assisted Scanning (SISAS) quantifies targeted peptides without retention time scheduling and with greater sensitivity than untargeted DDA approaches. The heavy standard and the matching endogenous peptide co-elute in the chromatogram but are resolved in the MS1 scan by a mass difference of +8 or +10 Da. Using the SureQuant feature on some Thermo mass spectrometers, when an MS1 precursor scan detects an m/z matching one of the heavy SIS peptides, a fast, low-resolution MS² scan event fragments the SIS precursor ion and matches its most abundant fragments to a list of known values. This confirms the identity of the SIS peptide and triggers high-sensitivity analysis of the matching endogenous peptide. Multiple fragment intensities are monitored as the peptide elutes, just as in a parallel-reaction-monitoring experiment. The areas under the peak curves are integrated in Skyline and the relative area changes can be calculated for each treatment condition.

SignalScan application to SARS-CoV-2 infected cell lines

Nucleocapsid protein



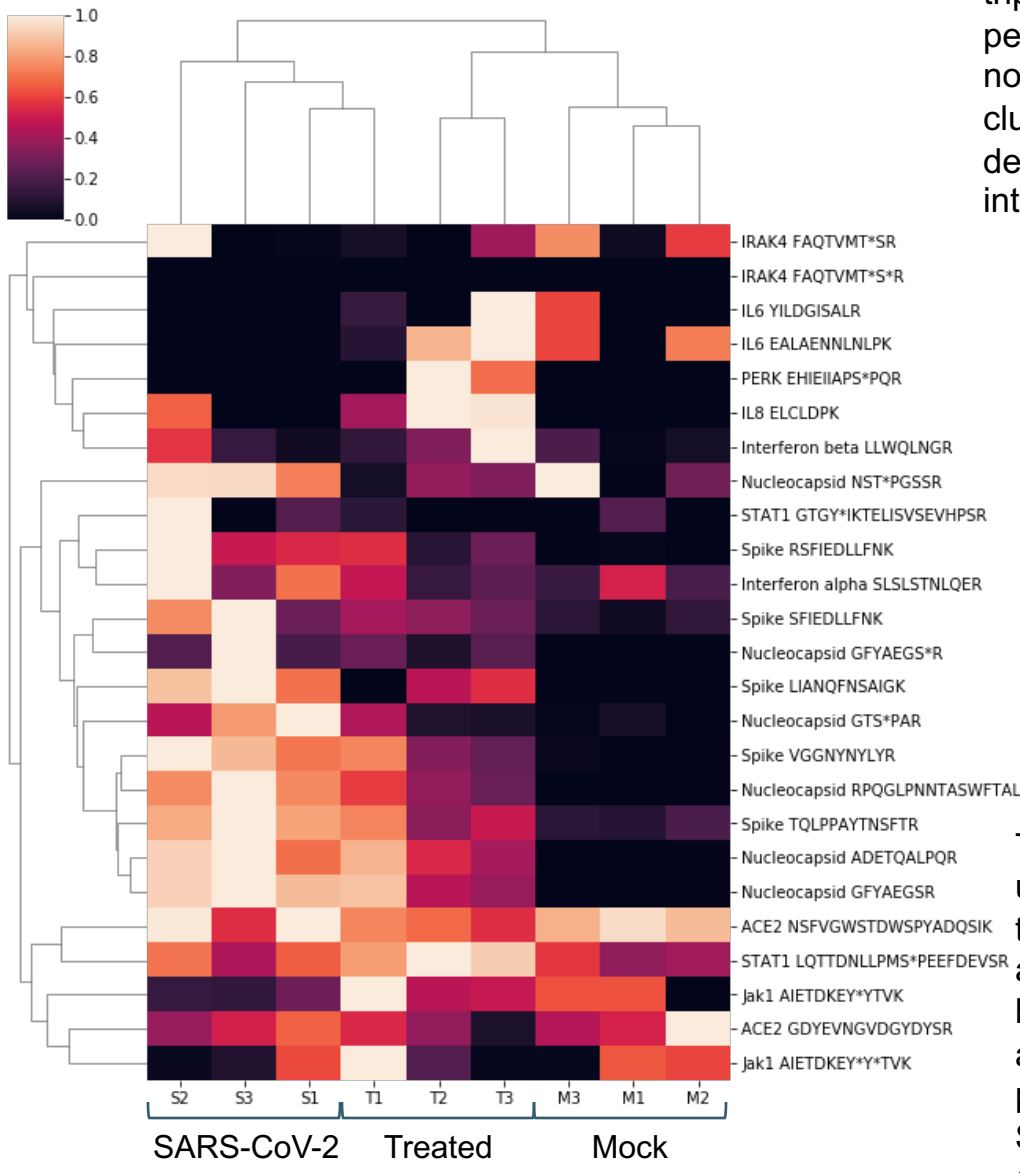
Spike protein



We applied the SignalScan SISAS workflow to two cell line systems used to study SARS-CoV-2 infection, human A549 cells over-expressing the ACE2 receptor and Vero cells, derived from the African Green Monkey (*Chlorocebus Sabaeus*). The cells were either mock-infected or infected with SARS-CoV-2, and infected cells were either untreated (labeled SARS-CoV-2) or treated with a kinase inhibitor. Independent measurements of different peptides from the same protein have consistent abundance ratios. As can be seen with both the Nucleocapsid and Spike proteins, viral load was reduced upon treatment in A549 cells but not in Vero cells.

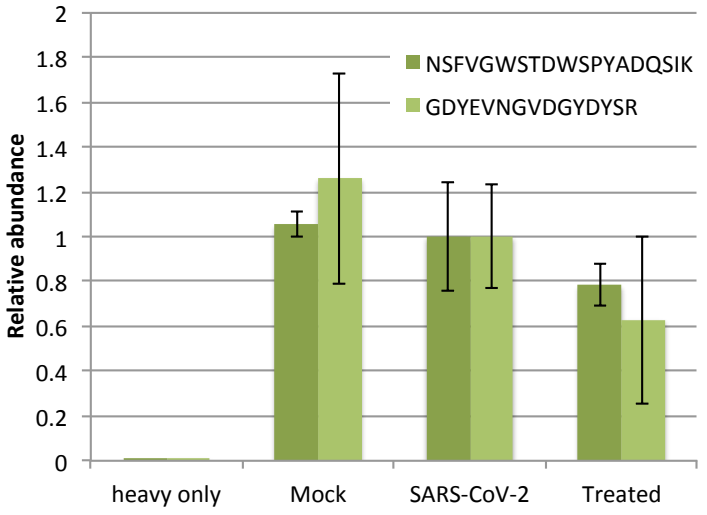
Experimental details: One pmol of each SIS peptide was spiked into one microgram of tryptic peptides derived from each condition, LCMS/MS was performed on a Fusion Lumos mass spectrometer using the SIS-Assisted Scanning (SISAS) method, and data analysis was performed in Skyline. The heavy SIS peptides were analyzed alone to determine any background signal. Abundance of each peptide was normalized to the average area under the curve (AUC) in the SARS-CoV-2 infected condition. Error bars represent standard deviations of independent biological triplicates.

A549 cell heatmap

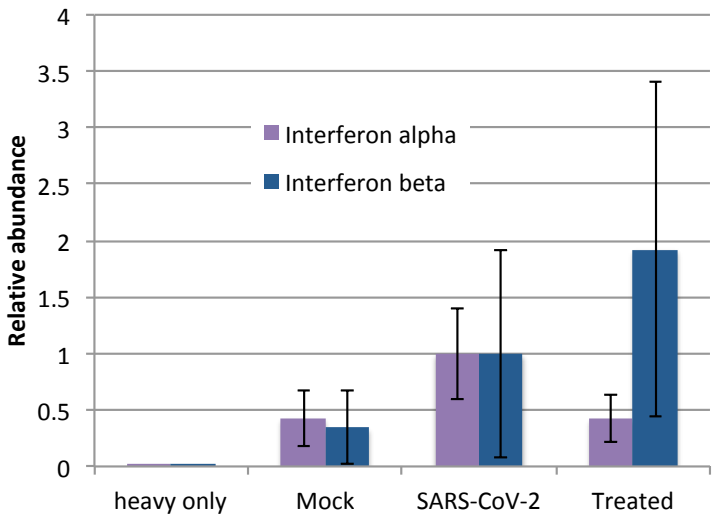


Left: Heatmap of peptide abundance of all SignalScan target peptides in A549 cells. Biological triplicates of different infection and treatment conditions cluster together, as do many related peptides, including those derived from viral proteins. For heatmap generation, raw intensities were normalized to the maximum observation among all samples for each peptide. Each sample was clustered using the unweighted pair group method with arithmetic mean (UPGMA) algorithm, and a dendrogram was generated from this clustering and plotted with a heatmap of normalized intensities.

ACE2 protein

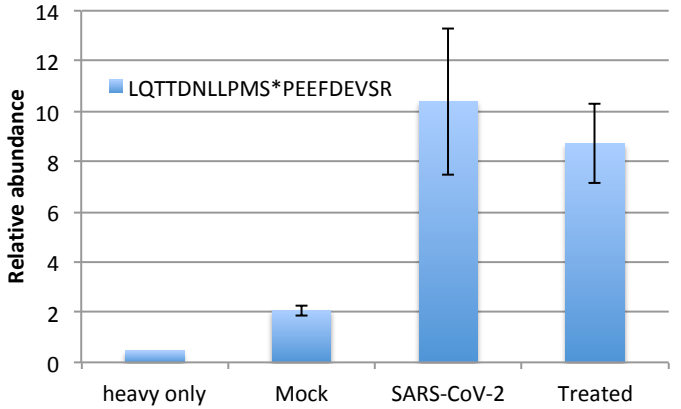


Interferon proteins

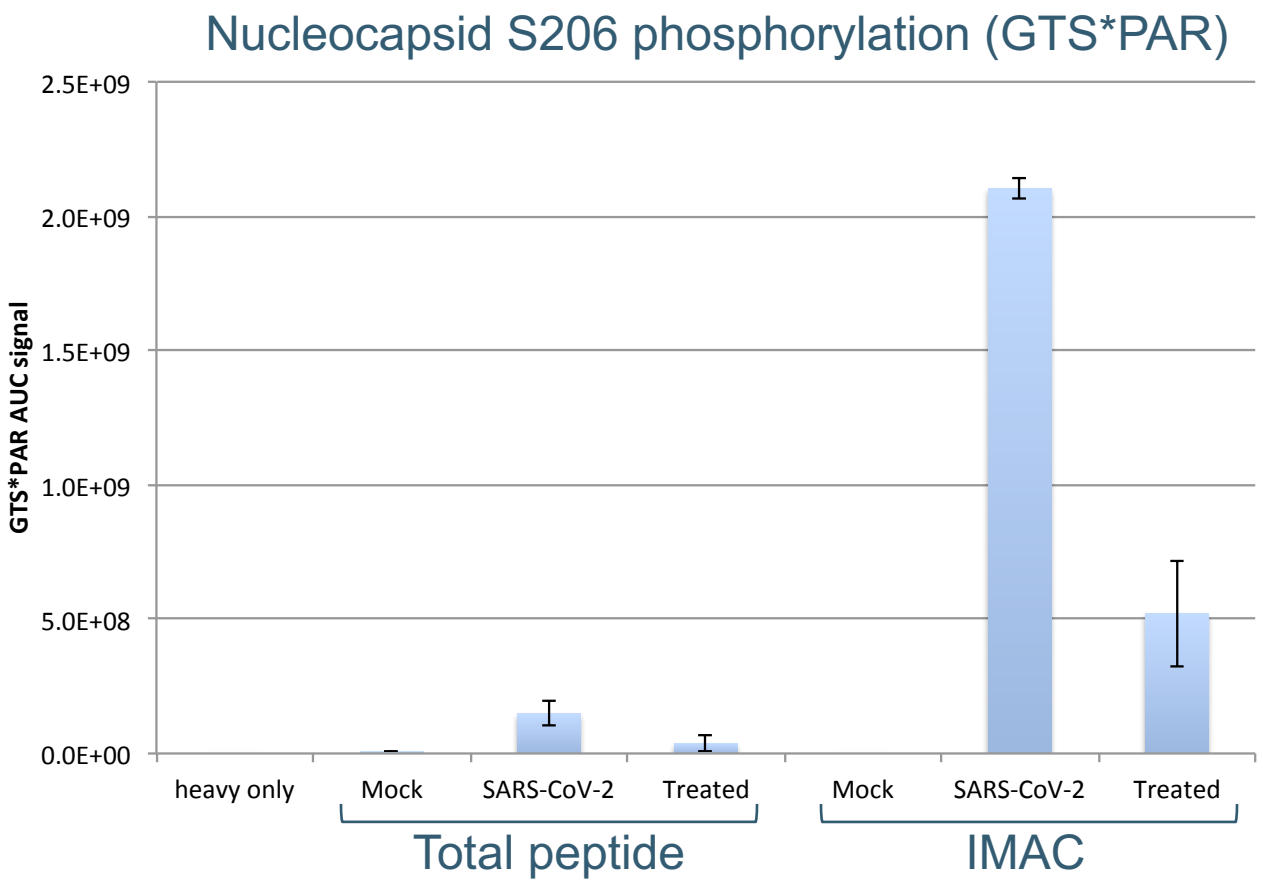


The levels of ACE2 protein are relatively unchanged in all conditions tested, potentially due to the overexpression system used. Interferon alpha and beta production in these cells seems to be induced upon viral infection, and the proteins are impacted differentially upon treatment. STAT1 phosphorylation at pS727 is also induced upon SARS-CoV-2 infection. This induction is most clearly observed when targeting the phosphopeptide in IMAC-enriched samples (right).

STAT1 pS727 phosphorylation



Using SignalScan to detect Nucleocapsid phosphorylation

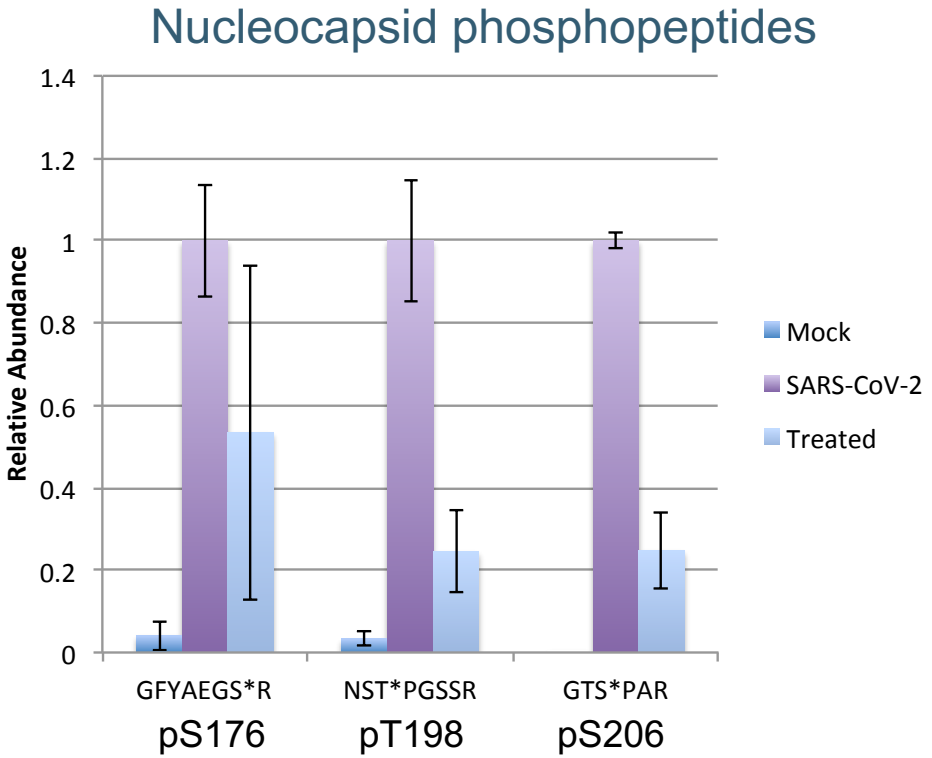


SignalScan targeting from **total peptide** sample:

- Simpler
- Enables analysis of all targets in a single LCMS run
- Preserves original relative abundance of all peptides for quantitation

Targeting from **phosphopeptide enriched** sample:

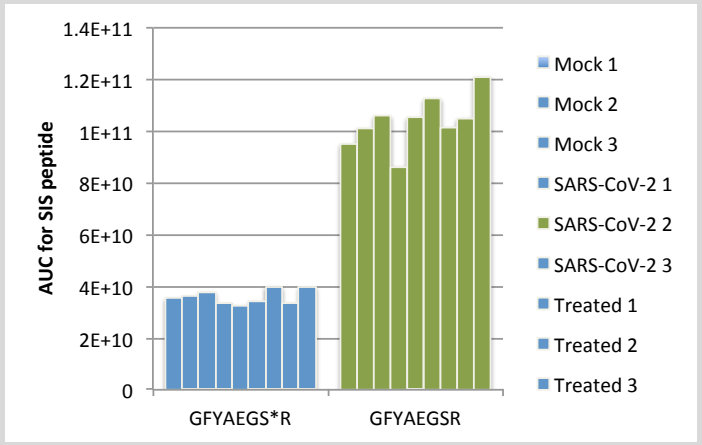
- Improves sensitivity for some sites
- Preserves intensity ratios between samples



Above: Intensity of Nucleocapsid pS206 phosphopeptide measured from total peptide (1.3 μg on column, left) or phosphopeptide (1% of IMAC-enriched peptide derived from 1 mg input total peptide right). Very similar profiles are observed using both sample preparation conditions, with reduction in intensity upon treatment clearly detectable, but sensitivity is improved with IMAC enrichment.

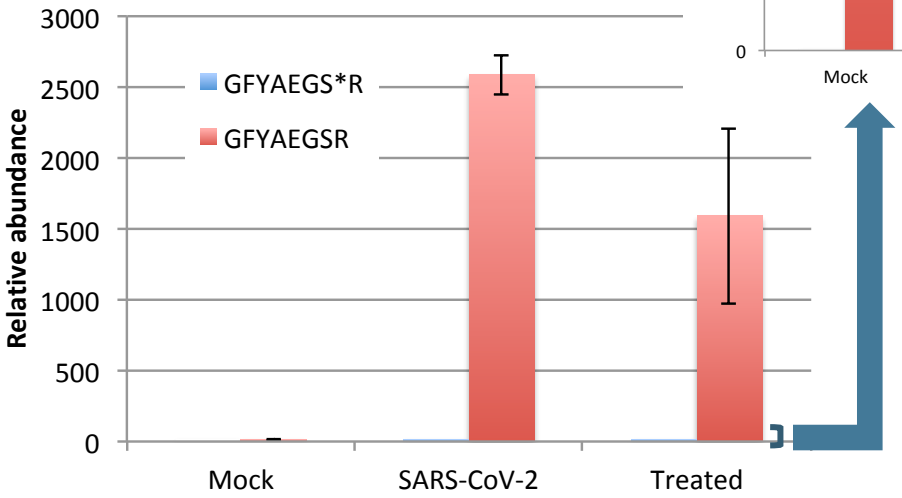
Right: Three sites of Nucleocapsid phosphorylation profiled from IMAC-enriched A459 samples, normalized to peptide level in SARS-CoV-2 infected cells. All three phosphopeptides have reduced abundance upon kinase inhibitor treatment.

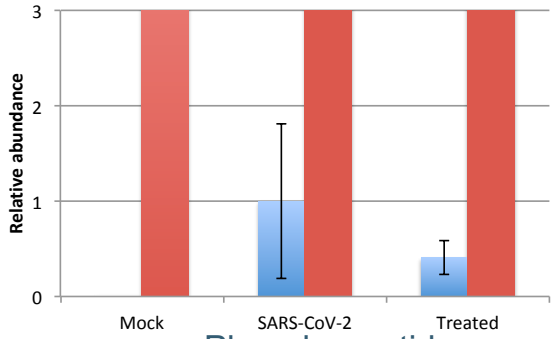
Using SignalScan Peptide Mix to assess stoichiometry of Nucleocapsid phosphorylation



Spiked SIS peptide signal for 1 pmol phosphorylated and unphosphorylated peptide across nine samples

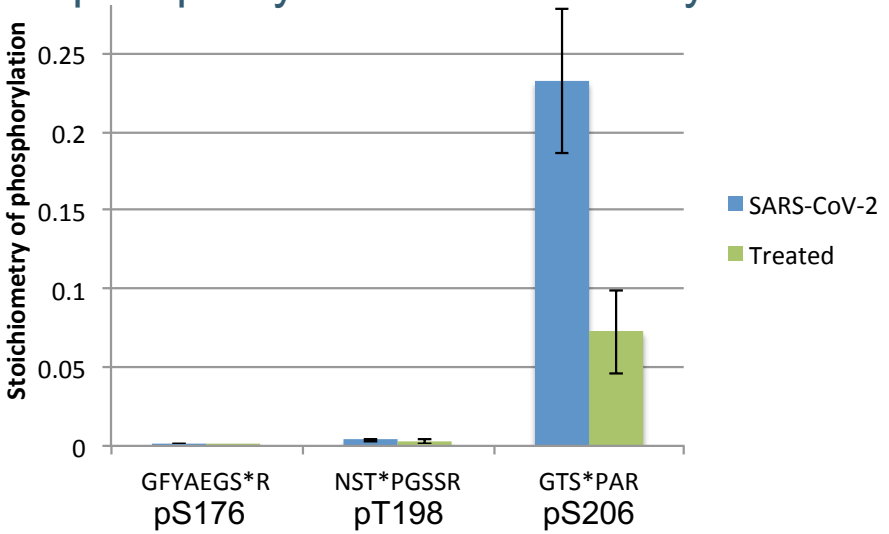
Normalize endogenous peptide signals to correct for differences in LCMS sensitivity





Phosphopeptide abundance (after correction for peptide sensitivity) is significantly less than abundance of unphosphorylated version of peptide, indicating stoichiometry of Nucleocapsid S176 phosphorylation is less than 0.1% in A549 cells

Site-specific Nucleocapsid phosphorylation stoichiometry



Right: We used the strategy depicted above to translate stoichiometry of phosphorylation to the other two targeted phosphorylation sites on the Nucleocapsid protein. Using the data from total peptide samples rather than IMAC (to prevent bias toward peptides more efficiently recovered by metal affinity), observed signal from SIS peptides was used to correct for LCMS sensitivity differences, and the summed signal from the GFYAEGSR peptide with and without phosphorylation was used as the total Nucleocapsid protein amount. This treatment of the data assumes equivalent production of these three phosphopeptides in the tryptic digestion and equivalent recovery from C18 peptide purification prior to LCMS, and therefore should be treated as an estimate. However, the striking difference in stoichiometry among these phosphorylation sites is likely to be significant even given those caveats. Ser206 is clearly the most highly phosphorylated site among the three. Additionally, though the total viral load is decreased upon kinase inhibitor treatment, we see that for all sites phosphorylation is reduced more than total protein, resulting in a decreased stoichiometry of phosphorylation upon treatment.

Using SignalScan to assess cleavage of Spike protein

SARS-CoV-2 Spike protein

MFVFLVLLPLVSSQCVNLTTRTQLPPAYTNSFTRGVYYPDKVFRSSVLHS
TQDLFLPFFSNVTWFHAIHVSGTNGTKRFDNPVLPFNDGVYFASTEKSNI
IRGWIFGTTLDSKTQSLLIVNNATNVVIKVCEFQFCNDPFLGVYYHKNNK
SWMESEFRVYSSANNCTFEYVSQPFLMDLEGKQGNFKNLREFVFKNIDGY
FKIYSKHTPINLVRDLPQGFSALEPLVDLPIGINITRFQTLLALHRSYLT
PGDSSSGWTAGAAAYYVGYLQPRTFLLKYNENGTITDAVDCALDPLSETK
CTLKSFTVEKGIYQTSNFRVQPTESIVRFPNITNLCPFGEVFNATRFASV
YAWNRKRISNCVADYSVLYNSASFSTFKCYGVSPTKLNDLCFTNVYADSF
VIRGDEVRQIAPGQTGKIADYNYKLPDDFTGCVIAWNSNNLDSKVGGNYN
YLYRLFRKSNLKPFERDISTEIYQAGSTPCNGVEGFNCYFPLQSYGFQPT
NGVGYQPYRVVVLSFELLHAPATVCGPKKSTNLVKNKCVNFNFNGLTGTG
VLTESNKKFLPFQQFGRDIADTTDAVRDPQTLEILDITPCSFGGVSVITP
GTNTSNQVAVLYQDVNCTEVPVAIHADQLTPTWRVYSTGSNVFQTRAGCL
IGAEHVNNSYECDIPIGAGICASYQTQTNSPRRARSVASQSIIAYTMSLG
AENSVAYSNNSIAIPTNFTISVTTEILPVSMTKTSVDCTMYICGDSTECS
NLLLQYGSFCTQLNRALTGIAVEQDKNTQEVFAQVKQIYKTPPIKDFGGF
NFSQILPDPSKPSKRSFIEDLLFNKVTLADAGFIKQYGDCLGDIAARDLI
CAQKFNGLTVLPPLLTDEMIAQYTSALLAGTITSGWTFGAGAALQIPFAM
QMAYRFNGIGVTQNVLYENQKLIANQFNSAIGKIQDSLSSTASALGKLQD
VVNQNAQALNTLVKQLSSNFGAISSVLNDILSRLDKVEAEVQIDRLITGR
LQSLQTYVTQQLIRAAEIRASANLAATKMSECVLGQSKRVDFCGKGYHLM
SFPQSAPHGVVFLHVTYVPAQEKNFTTAPAICHDGKAHFPREGVFVSNGT
HWFVTQRNFYEPQIITTDNTFVSGNCDVVIGIVNNTVYDPLQPELDSFKE
ELDKYFKNHTSPDVDLGDISGINASVVNIQKEIDRLNEVAKNLNESLIDL
QELGKYEQYIKWPWYIWLGFIAGLIAIVMVTIMLCCMTSCCSCLKGCCSC
GSCCKFDEDDSEPVLKGVKLHYT

S1 Domain
Receptor Binding Domain
S2 Domain
| = cleavage site

Uncleaved

…SKPSKRSFIEDLLFNKVTL…

RSFIEDLLFNK
(derived from uncleaved Spike)

Cleaved

SFIEDLLFNKVTL…

SFIEDLLFNK
(derived from both forms)

tryptic digestion

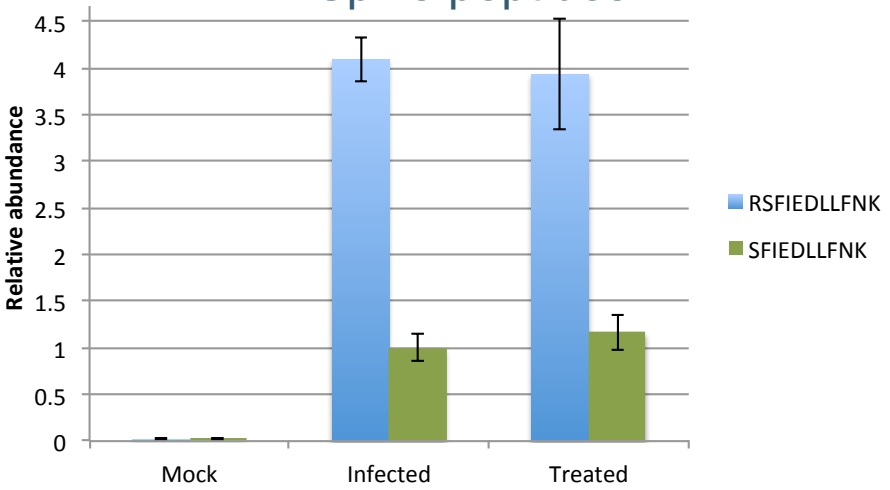
Spike protein is cleaved at two sites, the S1/S2 site and the S2' site, to mediate membrane fusion. The SignalScan Peptide Mix contains SIS peptides representing both cleaved (SFIEDLLFNK) and uncleaved (RSFIEDLLFNK) forms of the S2' site.

Because the biological cleavage event occurs at Arginine, this site is also a candidate for tryptic cleavage. Consequently, the SFIEDLLFNK peptide could be formed from either tryptic digestion or biological Spike cleavage. However the RSFIEDLLFNK peptide can ONLY be formed from Spike protein that was originally uncleaved in the biological sample. This peptide is commonly observed due to the adjacent Lysine at position 814 creating a ragged end cleavage pattern.

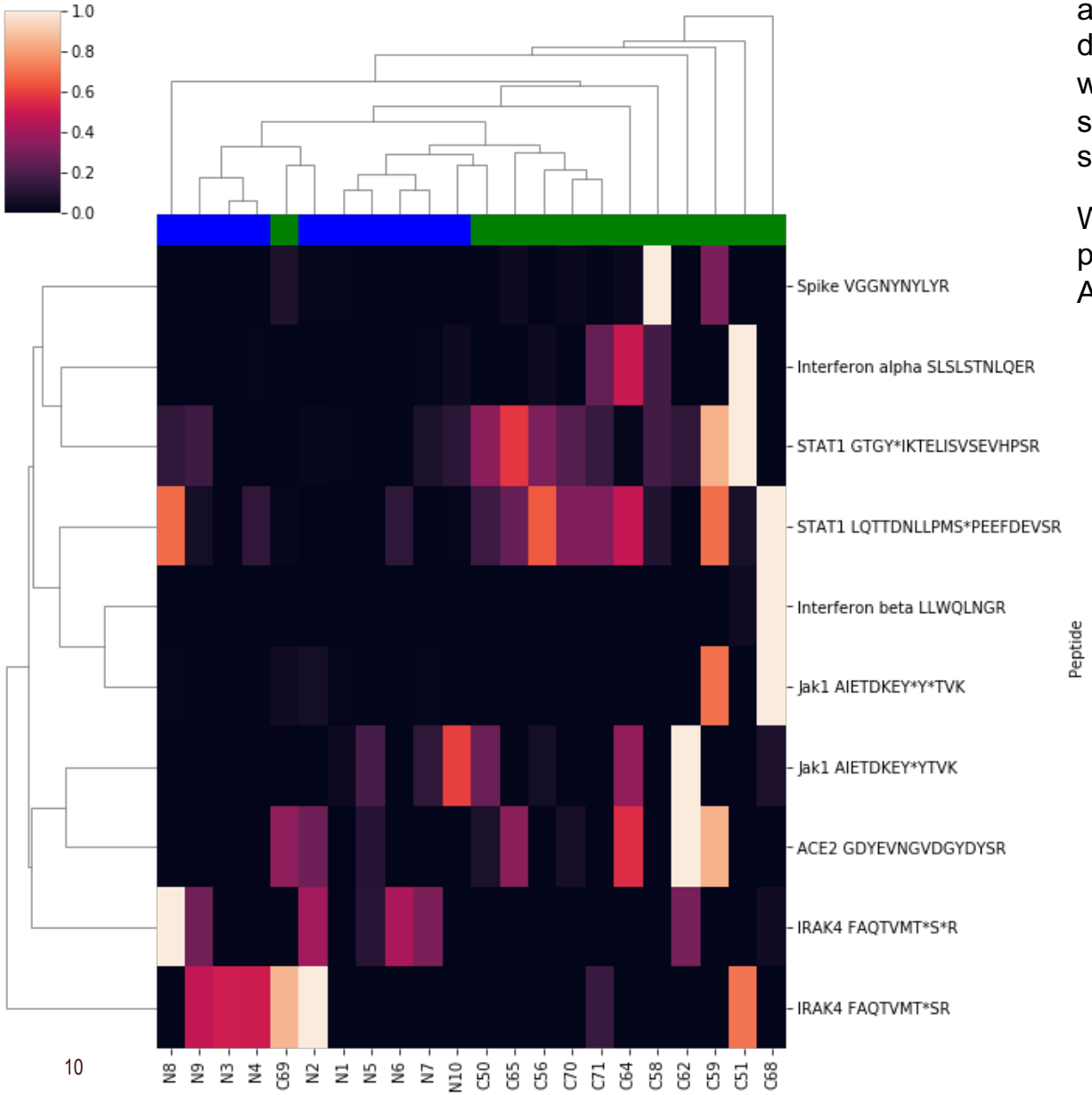
Right: In Vero cells, Spike protein is at least 80% uncleaved regardless of treatment condition.

As described on the previous slide for phosphorylation stoichiometry determination, the intensities of the spiked SIS peptides were used to correct for differences in LCMS sensitivity, enabling comparison of abundance between these related peptides. We detected 4x more of the uncleaved Spike peptide than the fully tryptic peptide, suggesting that at least 80% of the protein is in the uncleaved form.

Uncleaved vs. unknown cleavage Spike peptides



Human lung heatmap



Heatmap of peptide abundance of selected SignalScan target peptides in human lung samples from Covid-19 patients (labeled C50-C71, colored green at top of heatmap) and uninfected patients (labeled N1-N10, colored blue). Although the data are sparse due to protein degradation in the autopsy samples, some sites of phosphorylation were preserved, and the SignalScan targeted LCMS data were sufficient to produce separate clusters of the infected and uninfected patient samples except for a single sample reported to derive from a Covid-19 patient (C69).

We can see that IRAK4 phosphorylation is higher in normal samples while interferon production and Jak and STAT phosphorylation are stronger in infected patients. ACE2 expression also seems to be slightly elevated in Covid-19 patients.

Conclusions

- SignalScan Peptide Mix can be used to profile SARS-CoV-2 infection and treatment in human and related cell lines and tissues
- Peptides and phosphopeptides from both virus and host are monitored in highly sensitive, targeted LCMS assays without retention time scheduling
- IMAC can be used as desired to increase phosphopeptide sensitivity
- Utilizing quantified spiked Stable Isotope Standard peptides, researchers can investigate
  - Stoichiometry of Nucleocapsid phosphorylation
  - Fractional cleavage of Spike protein

Contact Kimberly Lee (klee@cellsignal.com) for additional information.

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