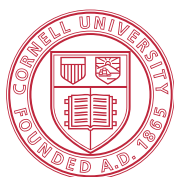


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Corresponding Author:	Marco Straus UNITED STATES
Corresponding Author's Institution:	
Corresponding Author E-Mail:	mrs393@cornell.edu
Order of Authors:	Javier A Jaimes Jean K Millet Monty E Goldstein Gary Whittaker Marco Straus
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Cornell University

College of Veterinary Medicine
Department of Microbiology & Immunology
Room C4 127 VMC, Ithaca NY 14853-3901
Tel: (+1) 607 253-4019 Fax: (+1) 607 253-3384
E-mail: mrs393@cornell.edu

August 1st, 2018

Dear Editor,

Please find enclosed a manuscript entitled "A fluorogenic peptide cleavage assay to screen for proteolytic activity on cleavage site motifs: Applications for coronavirus spike protein activation" by Javier A. Jaimes, Jean K. Millet, Monty E. Goldstein, Gary R. Whittaker and Marco R. Straus for submission of an original research article in the *Journal of Visualized Experiments*.

In our manuscript we present a novel and unique technique to test the proteolytic cleavage of peptide substrates by various proteases utilizing a rapid fluorogenic peptide cleavage assay. We demonstrate the benefits of this method by investigating the cleavage of peptides mimicking the cleavage site motif of viral fusion proteins and provide examples how the described fluorogenic peptide cleavage assay can assist the evaluation of potential public health risks posed by newly emerging viruses such as Middle East Respiratory Syndrome coronaviruses or influenza. However, the method is also of interest to other research investigating interactions between proteases and their proteolytic substrates.

We think our manuscript is of considerable interest, and would be a suitable and timely publication in the *Journal of Visualized Experiments*.

Sincerely,

A handwritten signature in black ink, appearing to read "Marco R. Straus".

Marco R. Straus

TITLE:

A Fluorogenic Peptide Cleavage Assay to Screen for Proteolytic Activity

AUTHORS AND AFFILIATIONS:

Javier A. Jaimes^{*1,2}, Jean K. Millet^{*1,3}, Monty E. Goldstein^{1,4}, Gary R. Whittaker¹ and Marco R. Straus¹

¹Department of Microbiology and Immunology, College of Veterinary Medicine, Cornell University, Ithaca, NY, USA

²Department of Microbiology, College of Agricultural and Life Sciences, Cornell University, Ithaca, NY, USA

³present address: INRA, Virologie et Immunologie Moléculaires, Domaine de Vilvert, Jouy-en-Josas, France

⁴Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, MD

*These authors contributed equally

Corresponding author:

Gary R. Whittaker (grw7@cornell.edu)

Tel: +1 607 253 4019

Email addresses of co-authors:

Javier A. Jaimes (jaj246@cornell.edu)

Jean K. Millet (jean.millet@inra.fr)

Monty E. Goldstein (meg362@umd.edu)

Marco R. Straus (mrs393@cornell.edu)

KEYWORDS:

Peptide assay, Coronavirus, Feline coronavirus, MERS-CoV, Fusion peptide, Protease cleavage, Furin

SUMMARY:

We present a fluorogenic peptide cleavage assay that allows a rapid screening of the proteolytic activity of proteases on peptides representing the cleavage site of viral fusion peptides. This method can also be used on any other amino acid motif within a protein sequence to test for the protease activity.

ABSTRACT:

Enveloped viruses such as coronaviruses or influenza virus require proteolytic cleavage of their fusion protein to be able to infect the host cell. Often viruses exhibit cell and tissue tropism and are adapted to specific cell or tissue proteases. Moreover, these viruses can introduce mutations or insertions into their genome during replication that may affect the cleavage, and thus can contribute to adaptations to a new host. Here, we present a fluorogenic peptide cleavage assay that allows a rapid screening of peptides mimicking the cleavage site of viral fusion proteins. The

technique is very flexible and can be used to investigate the proteolytic activity of a single protease on many different substrates, and in addition, it also allows exploration of the activity of multiple proteases on one or more peptide substrates. In this study, we used peptides mimicking the cleavage site motifs of the coronavirus spike protein. We tested human and camel derived Middle East Respiratory Syndrome coronaviruses (MERS-CoV) to demonstrate that single and double substitutions in the cleavage site can alter the activity of furin and dramatically change cleavage efficiency. We also used this method in combination with bioinformatics to test furin cleavage activity of feline coronavirus spike proteins from different serotypes and strains. This peptide-based method is less labor- and time intensive than conventional methods used for the analysis of proteolytic activity for viruses, and results can be obtained within a single day.

INTRODUCTION:

Viral fusion with the host cell membrane is a crucial step in the life cycle of enveloped viruses and facilitates entry into the cell and replication of the virus. Typically, viruses possess a specialized protein (or proteins) that binds to receptors on the host cell membrane and triggers the virus-cell membrane fusion¹. Viral fusion proteins have been grouped into three main classes (I-III)¹. A number of viruses that are currently a major concern for public health, such as influenza virus (representing a long-standing zoonotic emergence from birds) and Middle East Respiratory Syndrome-coronavirus (MERS-CoV) (representing a recent zoonotic emergence from camels), utilize the so-called class I fusion proteins, which require proteolytic processing by host proteases, to exert their fusogenic activity². Similarly, feline coronavirus (FCoV), which represents a major disease threat for wild and domestic cats, also possess a class I fusion protein. Class I viral fusion proteins are typically synthesized as an uncleaved precursor, and generally consist of two domains that are responsible for receptor binding and triggering the fusion event respectively. To date, the influenza hemagglutinin (HA) is the best understood fusion protein and numerous studies have described its mechanistic role in host cell entry and fusion³. In this case cleavage of the fusion protein occurs at a specific sequence or cleavage site within HA, and in combination with pH-dependent conformational changes, results in the exposure of the viral fusion peptide^{1,2}.

The fusion peptide and its preceding protease recognition sequence are critical for the pathogenicity and the host adaptation of the virus. Changes in the protease recognition sequence can alter cleavage significantly with potentially dramatic consequences for the virus and the host⁴. On one hand, it can abrogate cleavage and thus eliminate the life cycle of the virus. But on the other hand, a mutation can increase the substrate specificity for a given protease and/or allow a “new” protease to cleave the fusion protein. Subsequently, this can also expand the cell and tissue tropism as observed, *e.g.*, with influenza virus subtypes^{5,6}. Usually low pathogenicity avian influenza (LPAI) viruses and most human influenza viruses are confined to the gastrointestinal or respiratory tract because of the limited localization of the proteases that activate them. Typically, the fusion peptide of such HA proteins is preceded by a four-amino acid sequence that consists of 1-2 non-consecutive basic amino acids, which is recognized by trypsin-like serine proteases like trypsin, matriptase or TMPRSS2^{7,8}. When the virus acquires insertions or mutations that change the cleavage site to a polybasic site, it allows furin to activate the HA and to potentially cause a much more severe systemic infection^{6,9}. These viruses are referred to as high pathogenicity avian influenza virus (HPAI), typified by H5N1 strains.

89
90 In contrast to influenza HA, many coronaviruses, such as MERS-CoV, have two distinct cleavage
91 sites within their spike protein. The S1/S2 site separates the N-terminal receptor binding domain
92 (S1) from the C-terminal fusion domain (S2), with a second cleavage site, called S2', downstream
93 of the S1/S2 site, in proximity to the fusion peptide¹⁰. It was suggested that the sites are cleaved
94 sequentially, at S1/S2 followed by S2'. In contrast to the HA of most influenza virus strains, the
95 MERS-CoV S protein S1/S2 and S2' sites can be recognized by proteases of the proprotein
96 convertase (PC) family, such as furin. Members of this family cleave at paired basic residues with
97 the motif R/K-(X)_{0,2,4,6}-R/K(X, any amino acid)². Generally, the amino acids upstream of the
98 cleavage site are referred to as P1, P2, P3, *etc.* counting from the cleavage site and the amino
99 acids downstream are designated as P1', P2', P3', *etc.*¹¹. FCoV strains can either have a single or
100 a dual cleavage site. Like MERS-CoV, some FCoV strains also possess two cleavage sites (S1/S2
101 and S2') in their S protein. However, this characteristic is exclusive of serotype I FCoVs (clade A).
102 In contrast, members of the serotype II (clade B) grouping only have a single cleavage site (S2')^{2,12}.
103 Several proteases have been suggested to cleave the FCoV cleavage sites, including furin, trypsin-
104 like proteases and cathepsin. It has been proposed that the S protein of enteric FCoV (also known
105 as feline enteric coronavirus or FECV) is likely to be cleaved by furin in the S1/S2 site, and
106 mutations in this site (as well as at S2') leads to changes in protease requirements. These
107 mutations have been associated with changes in the tropism and pathogenicity of these viruses,
108 allowing the virus to become systemic and macrophage-tropic (also known as feline infectious
109 peritonitis virus or FIPV)¹³.

110
111 Viruses naturally introduce mutations into their genomes during each replication cycle and
112 frequently new subtypes and strains of influenza, MERS-CoV and FCoV are described¹⁴⁻¹⁶. As a
113 part of a rapid evaluation to assess the public health threat of emerging viruses, it is critical to
114 investigate changes in the cleavage site and how it affects the range of proteases activating these
115 viruses. Here, we describe a peptide-based assay that allows a very quick assessment of how the
116 cleavage site changes in MERS-CoV S protein affect the substrate specificity of a given protease
117 and to rapidly screen various proteases for their ability to cleave a given or multiple sequences⁴.
118 In a second set of experiments, we used the technique to determine the furin cleavage activity
119 over different FCoV serotypes and strains.

120
121 The peptides used in this assay are modified with the fluorescence resonance energy transfer
122 (FRET) pair, 7-methoxycoumarin-4-yl acetyl (MCA) at the N-terminus and N-2,4-dinitrophenyl
123 (DNP) at the C-terminus. During the assay, MCA is excited and emits light energy that is quenched
124 by DNP as long as the pair is in close vicinity to each other. If cleavage occurs, however, DNP is
125 not able to quench the emission anymore and it can be read by the fluorescence plate reader.
126 The changes in the fluorescence is measured during the experiment to determine the peptide
127 cleavage rate, and to calculate the velocity at which the protease cleaves the specific peptide
128 (also known as V_{max})¹⁷.

129
130 In order to design the peptides, the gene of the respective fusion protein must have been
131 sequenced and/or made available in a database. However, the method is less labor-intensive and
132 costly than conventional methods that usually require the cloning of the fusion protein gene into

expression vectors to express it in mammalian cells to analyze the cleavage. From start to end, this may take several days up to a few weeks while the peptide assays presented here can be done within one day as soon as peptides and proteases are available. The setup of the assay takes between 5 and 30 min depending on the number of samples and the runtime in the fluorescence plate reader is 1 h. Analysis of the data may take up to 2 h again depending on the sample size.

Here, we chose two different examples to present the assay. In the first example, we present data that compares the furin-mediated cleavage of human and camel-derived MERS-CoV, to assess the potential of the camel-derived strains of being activated in humans if they cross the species barrier. In the second example, we used a fluorogenic peptide assay to determine the furin-mediated cleavage of the S1/S2 and S2' sites or S2' site of two serotype I and two serotype II FCoV strains, respectively. For these experiments, we used trypsin cleavage as a positive control.

PROTOCOL:

1. Designing and Preparing the Peptides

1.1. Acquire the sequence of the fusion protein of interest from a public database such as NCBI (<https://www.ncbi.nlm.nih.gov/>) or the virus pathogen database (<https://www.viprbrc.org>). Choose the protease recognition site preceding the fusion peptide and include 2-3 amino acids upstream and downstream of this sequence.

1.2. When ordering the peptides, modify them with the FRET pair 7-methoxycoumarin-4-yl acetyl (MCA) at the N-terminus and N-2,4-dinitrophenyl (DNP) at the C-terminus.

Note: Several suppliers provide these modifications. Price and delivery time depend on the supplier of choice. However, alternative modifications exist which vary in sensitivity and require adjustments of the plate reader in terms of wavelengths.

1.3. Resuspend the peptide according to the manufacturers recommendations by gently pipetting up and down. For example, resuspend peptides in 70% ethanol to the final concentration of 1 mM.

1.4. Optionally, add the tube containing the peptide and the solvent into a sonication bath until it is fully resuspended if the peptide does not resuspend very well by pipetting.

1.5. Aliquot the peptide into light dampening or resistant tubes to protect the peptide from bleaching. Keep aliquot sizes small to avoid multiple freeze/thaw cycles (*e.g.*, 100 μ L). Store aliquots at -20° C.

2. Preparing the Fluorescence Plate Reader

2.1. Turn on the plate reader and wait until the self-test is finished.

2.2. Open the operating software on the attached computer and make sure it is connected with the plate reader.

2.3. Open the temperature setting and set to 30 °C (or the required temperature for optimal performance for the protease).

2.4. Click on **Control | Instrument Setup** to set up the experiment.

2.5. Choose Kinetic.

2.6. Select Fluorescence.

2.7. Enter Excitation and Emission wavelengths: 330 nm and 390 nm respectively.

2.8. Unselect Auto Cut-off.

2.9. Select Medium, normal sensitivity.

2.10. Choose a runtime of 1 h for the assay and select one measurement every 60 s.

2.11. Set 5 s mixing before first measurement and 3 s before each measurement

2.12. Select wells to read and start the assay.

3. Preparing the Assay

3.1. Prepare the assay buffer by calculating the appropriate quantities for each ingredient and adding it. For furin, make buffer consisting of 100 mM HEPES, 1 mM CaCl₂, 1 mM 2-mercaptoethanol, 5% Triton X-100. For trypsin, use standard phosphate buffered saline (PBS) solution.

Note: Volumes of 10 mL or less are sufficient. Depending on the protease(s) used in the assay the buffer varies.

3.2. Chill the buffer on ice.

3.3. Place the assay plate on ice with a thin metal plate underneath to support cooling and stability. Use a solid black polystyrene 96-well plate with a flat bottom and non-treated.

Note: It is essential to use black plates to prevent fluorescent “leakage” from adjacent wells.

3.4. Calculate the appropriate amount of protease to add for each reaction based on previous publications or experimental data. For furin, use 1 unit per reaction which corresponds to 0.5 µL.

For trypsin, use 0.5 μ L of 160 nM TPCK trypsin.

3.5. Per sample prepare 3 technical replicates per assay in a total volume of 100 μ L per sample.

Note: It was experimentally evaluated that it does not make a difference whether the pipetting is carried out under normal light conditions or dimmed light. However, the peptides should not be exposed to light for an extended period of time.

3.6. Pipette the appropriate amount of assay buffer (94.5 μ L as described under Step 3.1) into each well.

3.7. Add the protease to each well. For both, furin and TPCK trypsin, use 0.5 μ L per sample. To 6 wells (3 wells for a blank control and 3 wells for a peptide control), add 0.5 μ L buffer instead of the respective protease.

3.8. Add the peptide to a final concentration of 50 μ M to each well except the blank control. In this case, pipette 5 μ L peptide prepared as described under Step 1.3. Add 5 μ L of buffer to the blank control instead of peptide.

Note: Several concentrations of the peptide were initially tested with the described enzyme concentrations to ensure that the enzymes were saturated.

3.9. Insert the plate into the fluorescence plate reader and click start.

4. Data Analysis

4.1. Save the experiment file.

4.2. Click on **Export** and export the file as a .txt.

4.3. Import the .txt file into a spreadsheet.

4.4. For each technical replicate per sample, create a graph plotting the relative fluorescent units (RFU) on the y-axis against the time on the x-axis (**Supplemental Figure 1**).

4.5. Select the data range where the graph is in a linear range and the closest to the start of the fluorescence increase.

4.6. Plot the selected data on a second graph and add a linear trendline.

4.7. Select in the trendline options **Display equation on chart**. The equation will show the Vmax.

4.8. Calculate the average Vmax for each sample from the 3 technical replicates.

4.9. Repeat the experiment twice more to obtain 3 biological replicates.

4.10. Calculate the standard deviation based on the data from 3 independent biological replicates.

REPRESENTATIVE RESULTS:

In the first part of this study, we used peptides that represent the S2' cleavage site of three distinct MERS-CoV S proteins: from the prototypical EMC/2012 human strain (Genbank AFS88936.1) and from two selected camel-derived MERS-CoVs, NRCE-HKU205 (Genbank AHL18090.1) and Mor215 (Genbank AVN89324.1) strains, isolated in Egypt and Morocco, respectively (**Table 1**)^{16,18}.

Compared to the EMC/2012 strain, the HKU205 S2' cleavage site has two mutations in the P2 and P1' positions that could potentially impact its recognition by furin and alter the cleavage efficiency (**Table 1**). In addition, we were interested to investigate if and how each individual mutation found in the HKU205 strain affects furin cleavage. We, therefore, included two peptides where the individual substitutions were introduced into the EMC/2012 peptide sequence (EMC/2012 mutA-S S2' and EMC/2012 mutS-I S2') (**Table 1**). Furin was able to cleave the EMC/2012 peptide efficiently with a Vmax of 6.3 ± 1.2 RFU/min, while we detected almost no cleavage when using the S2' peptide of the HKU205 strain ($V_{\max} 0.5 \pm 0.1$ RFU/min) (**Figure 1a**). When investigating the EMC/2012 mutA-S S2' peptide, we found that the cleavage was strongly reduced compared to the wild type sequence ($V_{\max} 3.6 \pm 1$ RFU/min). There was almost no cleavage when testing the EMC/2012 mutS-I S2' peptide ($V_{\max} 1.1 \pm 0.9$ RFU/min) (**Figure 1a**).

Recently, MERS-CoV isolates from Western Africa were reported that are phylogenetically distinct from MERS-CoV found in the Arabian Peninsula¹⁶. One of the recently described isolates is the Mor213 strain, which carries a leucine instead of an arginine in the P4 position of the S2' site (**Table 1**), and which could potentially impact its recognition by furin and alter the cleavage efficiency. Our peptide assay showed that there is only minimal cleavage of the Mor213 S2' site as compared to the EMC/2012 site (**Figure 1b**). The Vmax for the Mor213 peptide is 1.0 ± 0.1 .

For the second part of this study, we used the same peptide assay to evaluate furin-mediated cleavage of FCoV S protein cleavage sites. We used peptides mimicking the S1/S2 cleavage site of two FCoV serotype I viruses: FECV I And-4 (from Whittaker lab) and FIPV I Black (Genbank AB088223.1). We also used peptides mimicking the S2' site of these viruses, as well as two FCoV serotype II: FECV II 1683 (Genbank AFH58021.1) and FIPV II 1146 (Genbank AAY32596.1) strains (**Table 1**). Typically, furin cleavage occurs when basic residues (arginine and lysine) are present in the P1 and P4 positions of the cleavage site. Mutations in the P1, P4 and P1' positions are suggested to interfere with furin cleavability, therefore altering the protease requirements of the protein. (**Table 1**). Furin cleavage was observed for the prototypical S1/S2 peptide FECV I And-4 S1/S2 ($V_{\max} 100.36 \pm 7.61$ RFU/min) but not in the mutated S1/S2 peptide FIPV I Black S1/S2 ($V_{\max} -1.37 \pm 1.29$ RFU/min) (**Figure 2A**). Similarly, furin was able to cleave the prototypical S2' peptide FECV II 1683 S2' ($V_{\max} 5.46 \pm 0.97$ RFU/min), but not the mutated S2' peptides FECV I And-4 S2' ($V_{\max} 0.26 \pm 0.11$ RFU/min), FIPV I Black S2' ($V_{\max} 0.30 \pm 0.14$ RFU/min) and FIPV II

1146 S2' ($V_{\max} -0.36 \pm 0.11$ RFU/min) (**Figure 2B**). We used trypsin as a positive control for peptide cleavage and we observed trypsin cleavage in all the peptides used (**Supplemental Figure 2**).

FIGURE AND TABLE LEGENDS:

Table 1. Peptides used and corresponding amino acid sequences. The peptides used in the assays contain the (7-methoxycoumarin-4-yl) acetyl/2,4-dinitrophenyl (MCA/DNP) FRET pair. The P4 and P1 cleavage site positioned arginine (R) residues, which are recognized by furin, are in bold. Residues in red correspond to mutations compared to reference sequences.

Figure 1. Furin-mediated proteolytic cleavage of human and camel-derived MERS-CoV S2' sites fluorogenic peptides. **A.** Furin cleavage assay of human MERS-CoV EMC/2012 S2' site and camel-derived strain HKU205 (double mutant), along with single mutant variants of EMC/2012 (mutA-S and mutS-I). **B.** Furin cleavage assay of human MERS-CoV EMC/2012 S2' site and camel-derived strain Mor213. For panels **A** and **B**, peptides were incubated with recombinant furin and the increase in fluorescence due to proteolytic processing was measured using a fluorometer. The assays were performed in triplicates with results representing averages of V_{\max} from three independent experiments ($n=3$). Error bars indicate SD.

Figure 2. Furin-mediated proteolytic cleavage of FCoV S1/S2 and S2' sites fluorogenic peptides. **A.** Furin cleavage assay of FECV I And-4 and FIPV I Black S1/S2 sites. Peptides were incubated with recombinant furin. **B.** Furin cleavage assay of FECV I And-4, FIPV I Black, FECV II 1683 and FIPV II 1146 S2' sites. The increase in fluorescence due to proteolytic processing was measured using a fluorometer. The assays were performed in triplicates with results representing averages of V_{\max} from three independent experiments ($n=3$). Error bars indicate SD.

Supplemental Table 1: V_{\max} values used for Figures 1 and 2. The V_{\max} values were calculated from graphs as illustrated in Supplemental Figure 1 and as described in Protocol section 4.

Supplemental Figure 1: Illustration of raw data derived from the plate reader software. Increase of fluorescence over time graphs that were used for determination of the V_{\max} .

Supplemental Figure 2: Trypsin-mediated proteolytic cleavage of FCoV S1/S2 and S2' sites fluorogenic peptides. Trypsin cleavage assay of FECV I And-4 and FIPV I Black S1/S2 sites and of FECV I And-4, FIPV I Black, FECV II 1683 and FIPV II 1146 S2' sites.

DISCUSSION:

We present here a fluorogenic peptide assay that allows for a fast screening of protein sequences for their proteolytic cleavage by proteases. In our first example we chose different cleavage site motifs of the MERS-CoV spike (S) protein to illustrate one application for this assay. Several enveloped viruses that possess class I fusion proteins such as MERS-CoV, SARS-CoV (Severe Acute Respiratory Syndrome) and influenza are major concerns for public health and new subtypes are constantly evolving that have a potential to cross species barriers from their natural hosts to

humans^{1,15,16}. Isolating the viruses and cultivating them in the lab may not always be feasible or requires special laboratories that are not available in every research facility. Hence, there is a need for methods to assess the potential public health threat of emerging viruses that can be conducted under regular laboratory conditions. In addition to viruses targeting humans, some animal viruses like FCoV also possess the same class I fusion proteins, therefore, sharing the similar characteristics than their human counterparts. Isolating these viruses can also be a challenge, making the use of innovative tools to study them necessary.

A crucial step in the life cycle of the above-mentioned viruses is host cell entry and fusion mediated by proteolytic processing of the respective fusion protein by host proteases^{1,2}. A standard way to investigate this part of the viral life cycle is to clone the fusion protein gene into a mammalian expression vector, transfect mammalian cells that allow for protein expression, incubate or co-transfect with the protease of interest, isolate the protein and perform a western blot analysis. This method comes with several limitations: availability of viral DNA/RNA for the cloning, costs for synthesizing the gene if no DNA or RNA is available, availability of an antibody to detect the fusion protein and its cleavage product(s), and it can take up to several weeks until the entire study is performed. It even becomes more time-consuming, money and labor intensive if the interest of the study is to investigate several mutations in the cleavage site because it requires site-directed mutagenesis. The fluorogenic peptide assay we describe here does not have these limitations. The peptides of interest can be designed based on publicly available sequences, there are several suppliers that provide a wide range of recombinant proteases that are relevant for these kinds of studies and the assay including the analysis can be performed within a single day.

In our example, we examined furin-mediated cleavage of the S2' protease recognition site of the MERS-CoV S protein derived from humans and camels from Egypt and Morocco. Both the human EMC/2012 and the camel HKU205 S2' sites contain a typical RXXR furin cleavage motif. However, according to the PiTou 2.0 cleavage scoring algorithm the HKU205 S2' site is not predicted to be cleaved by furin, which we experimentally confirmed¹⁹. The reason why HKU5 S2' is not processed by furin might be due to the isoleucine in the P1' position. When we tested two peptides that carried the individual mutations in the EMC/2012 S2' sequence, we were able to confirm that the isoleucine in the P1' largely abrogates cleavage whereas the alanine to serine substitution resulted in reduced cleavage. The Mor213 S2' site does not contain a furin cleavage motif and it was not surprising to detect almost no cleavage. We also examined how furin cleaves the S1/S2 and the S2' protease recognition sites of the FCoV S protein. We were able to observe furin cleavage of both FECV peptides (FECV I And-4 S1/S2 and FECV II 1683 S2'), while mutated sequences from FIPV viruses were not cleaved by furin.

When comparing furin-mediated cleavage of the EMC/2012 S2' peptide (**Figure 1A**) with the FECV I And-4 S1/2 peptides (**Figure 2A**), we found that there was an approximate 26-fold difference in the Vmax. This can be explained by the furin cleavage motif in both sequences (**Table 1**). While the minimum requirement for furin cleavage is a RXXR motif, a RXRR sequence is much more favorable². Therefore, the EMC/2012 S2' peptide, which has a RSAR motif, is cleaved with less specificity compared to the FECV I And-4 S1/2 peptide that contains a RSRR furin cleavage site

(Table 1).

However, one major limitation of the assay is that the peptides do not reflect the tertiary structure of the protein they are usually embedded in. Cleavage of the full length fusion protein exposes the fusion peptide and triggers host cell entry¹. The interaction between protease and fusion protein might also be impacted by the tertiary structure of the fusion protein while we assume that the peptides are more or less linear. Hence, cleavage detected in the peptide assay may be artificial and may not reflect the *in vivo* situation. This was reported for cleavage of the influenza H3N2 HA subtype by matriptase. While several groups described cleavage of the H3N2 HA by matriptase in peptide assays, it was also shown that incubation of full length H3N2 HA protein and matriptase in cell culture did not result in a fusogenic HA protein^{4,20,21}. There are other examples that show that the biologically important regulation of the proteolytic cleavage is on the level of protein conformation. It has been described that fusion proteins sometimes need a series of pre-fusion events to be able to expose the cleavage sites upon fusion. The Semliki Forest virus fusion protein, for example, requires structural rearrangements during a number of prefusion events in order to expose its fusion protein and make it available for proteolytic activation²². Therefore, the results obtained in a fluorogenic peptide assay need to be validated by cell fusion assays or similar experiments. However, the peptide assay still allows a fast screening of several protease/peptide combinations and to reduce the labor and time of follow up experiments since combinations that do not show cleavage are very likely to exhibit the same result *in vivo*.

The second major limitation of the assay concerns the proteases. So far it is only possible to test soluble proteases but not transmembrane proteases. Depending on the intent of the experiment, this can be a problem. For example, influenza HAs are activated by a number of transmembrane proteases located at cell membranes⁸. To a certain extent, this problem can be circumvented by using the soluble proteolytic active domains, but the results have to be interpreted with caution and should be validated with conventional methods. We want to refer to the beforementioned example with matriptase and its activity towards H3N2 HA. *In vivo* matriptase is located in the cell membrane but the commercially available enzyme is the soluble catalytic domain. As discussed with respect to the fusion proteins, it may be possible that it also requires the full-length protease to interact with the full-length protein substrate to obtain cleavage and that testing only single domains may result in false positives.

This methodology has shown to be efficient to study the cleavage of specific amino acid sequences by furin. However, the applications of the technique extends to any available protease (*e.g.*, cathepsins, proprotein convertase), making it a useful method to screen peptide candidates for protease cleavage. Moreover, it has been shown that this assay can also be used to test the efficacy of protease inhibitors and hence provides a fast and easy to tool to screen protein inhibitors²³.

The fluorogenic peptide cleavage assay described here represents an addition to bioinformatic cleavage scoring algorithms, which predicts the peptide cleavage by a determined protease, based on the amino acid properties and sequence. These two techniques, in combination with

tradition western blotting techniques can be used as an efficient method to study protease cleavage *in vitro*.

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DISCLOSURES:

The authors have nothing to disclose.

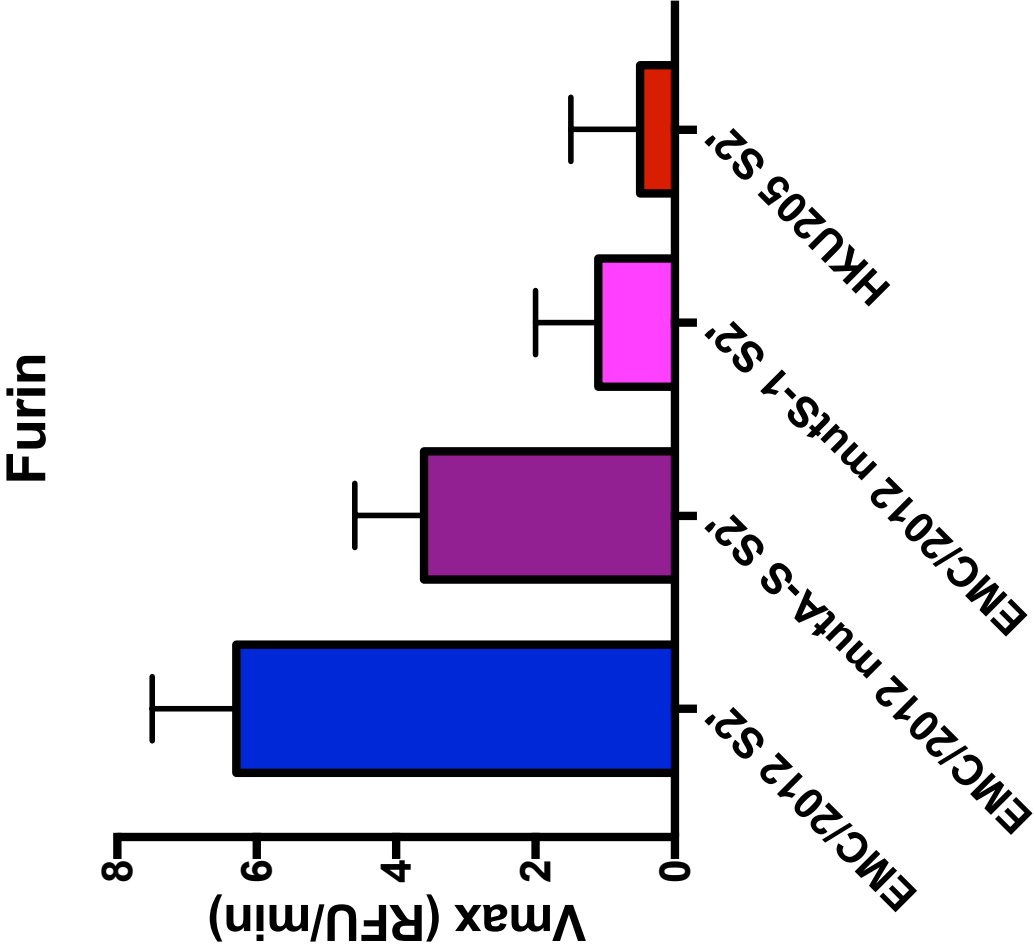
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Figure 1

A



B

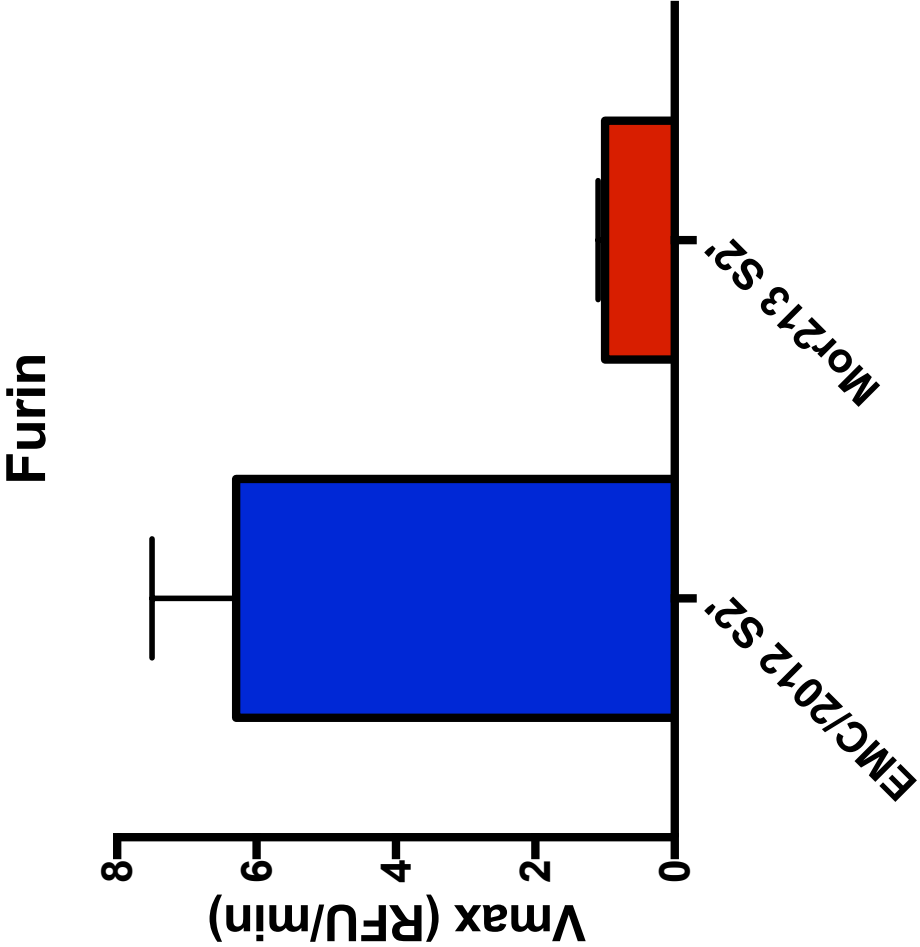
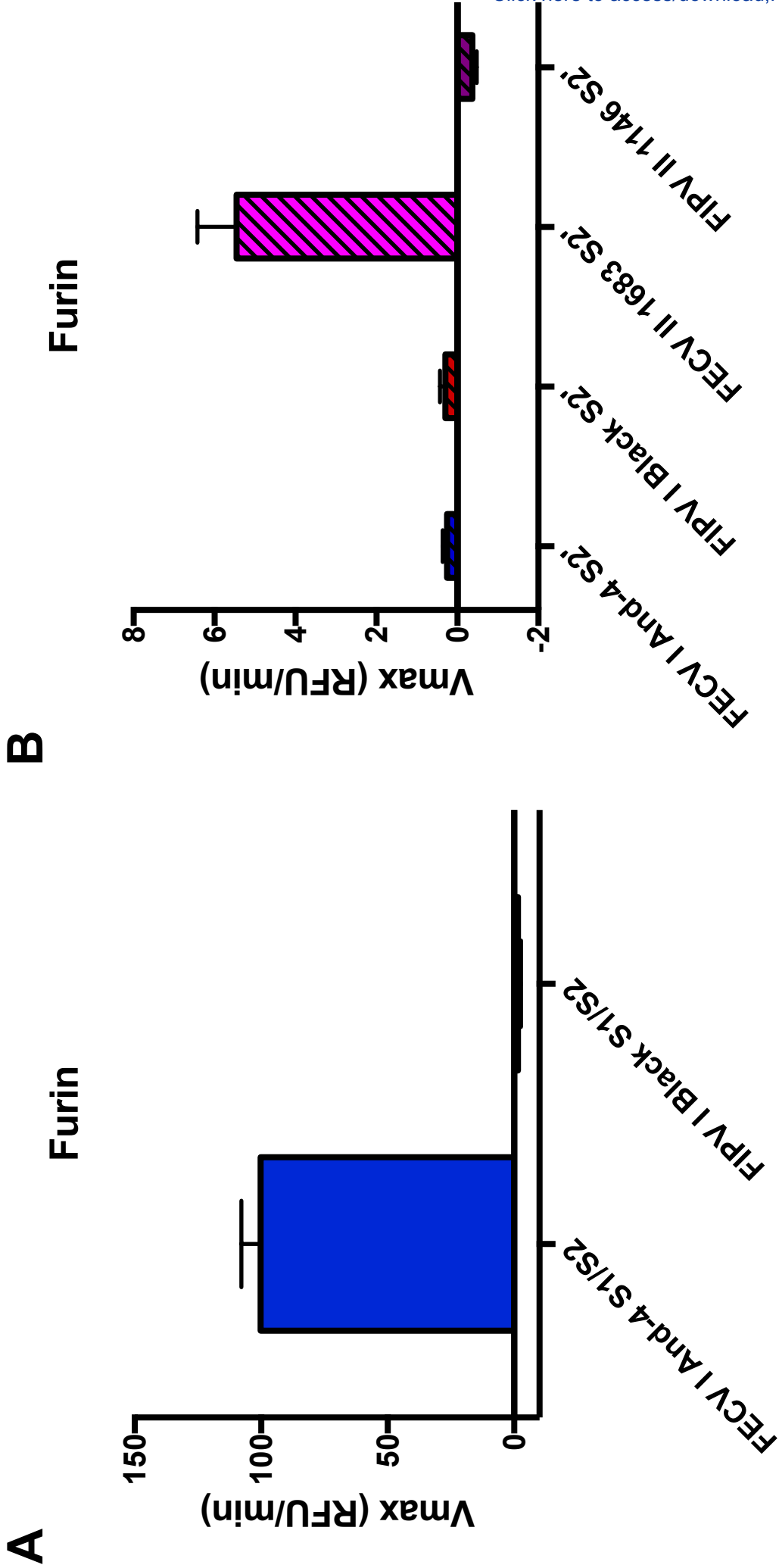


Figure 2

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Name	Sequence						
	N-terminus	P4	P3	P2	P1	P1'	C-terminus
MERS-CoV EMC/2012 S2'	(MCA)-GS	R	S	A	R	S	AIE-(DNP)
MERS-CoV EMC/2012 mutA-S S2'	(MCA)-GS	R	S	S	R	S	AIE-(DNP)
MERS-CoV EMC/2012 mutS-1 S2'	(MCA)-GS	R	S	A	R	I	AIE-(DNP)
MERS-CoV HKU205 S2'	(MCA)-GS	R	S	S	R	I	AIE-(DNP)
MERS-CoV Mor213 S2'	(MCA)-GS	L	S	A	R	S	AIE-(DNP)
FECV I And-4 S1/S2	(MCA)-TSSR	R	S	R	R	S	TT-(DNP)
FIPV I Black S1/2	(MCA)-TQAK	R	S	R	R	P	TS-(DNP)
FECV I And-4 S2'	(MCA)-PT	I	G	K	R	S	AVE-(DNP)
FIPV I Black S2'	(MCA)-PT	I	G	V	R	S	AVE-(DNP)
FECV II 1683 S2'	(MCA)-SK	R	K	Y	R	S	AIE-(DNP)
FIPV II 1146 S2'	(MCA)-SK	R	K	Y	G	S	AIE-(DNP)

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Peptides	Biomatik	N/A	
Furin	NEB	P8077S	
Trypsin, TPCK-treated	Sigma-Aldrich	4352157-1KT	
HEPES	Sigma-Aldrich	H3375	
CaCl ₂	Sigma-Aldrich	C1016	
2-Mercaptoethanol	Sigma-Aldrich	M6250	
Triton-X100	Sigma-Aldrich	X100	
PBS	Corning	21-040-CV	
SpectraMax Gemini XPS	Molecular Devices	XPS	
SoftMax Pro 6.5.1	Molecular Devices	N/A	
96-well plate (solid black polystyrene)	Costar	3915	
Light damping tubes	Watson Lab	131-915BL or 131-915BR	
Microsoft Excel	Microsoft	N/A	
Prism 7	GraphPad	N/A	



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A fluorogenic peptide cleavage assay to screen for proteolytic activity on cleavage site motifs: Applications for coronavirus spike protein activation

Author(s):

Javier A. Jaimes, Jean K. Millet, Monty E. Goldstein, Gary R. Whittaker¹ and Marco R. Straus

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Name:

Gary Whittaker

Department:

Microbiology and Immunology


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Changes to be made by the Author(s) regarding the manuscript:

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[The revised version of the manuscript has been proofread.](#)

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[The numbering of the protocol has been adjusted.](#)

5. Please revise the protocol text to avoid the use of any personal pronouns (e.g., “we”, “you”, “our” etc.).

[We removed all personal pronouns.](#)

6. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

[The protocol has been revised for the addressed issues.](#)

7. Line 179: Please write the text in the imperative tense in complete sentences.

[Edited.](#)

8. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

[We revised the protocol and tried to be more clear and detailed. If there are steps that still seem to require to be more detailed, we are happy to provide the descriptions. However, we would appreciate if it was specifically pointed out which steps need revisions in such a case.](#)

9. Please include single-line spaces between all paragraphs, headings, steps, etc.

[Changed in manuscript.](#)

10. References: Please do not abbreviate journal titles.

[Respective journal titles in the references were changed.](#)

11. Please revise the table of the essential supplies, reagents, and equipment to include the name, company, and catalog number of all relevant materials.

[Table has been revised.](#)

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In this manuscript entitled "A fluorogenic peptide cleavage assay to screen for proteolytic activity on cleavage site motifs: Applications for coronavirus spike protein activation", authors describe using a fluorogenic peptide cleavage assay with different amino acid substitutions from different viral strains on the peptide substrate containing FRET pair. Assay revealed different V_{max} of these peptide substrates. This reviewer has the following concerns or questions to be answered.

Major Concerns:

1. They have measured V_{max} values of different fluorogenic peptides cleaved by Furin. However, by definition, V_{max} is the maximal activity with a saturated amount of substrate (at least 5-fold of K_m). From the description, they did not use different substrate concentrations (0.5-5-fold K_m) to determine the V_{max} . They only used peptide substrates to a final concentration of 50 μM . Is that concentration sufficient to saturate the enzyme? They probably should measure the initial rates under different substrate concentrations to obtain K_m and V_{max} , so they can also discuss about the K_m (substrate affinity). If they are sure 50 μM is high enough (please explain), they can claim what they measured are the V_{max} .

We thank the reviewer for the important points raised. As suggested by the reviewer, we previously performed experiments in which we measured the initial rates under different peptide substrate concentrations and found that 50 μM of peptide was indeed sufficient to saturate the enzyme. These preliminary experiments showed that at concentrations of peptide substrate higher than 50 μM , there were negligible effects on initial rates or proteolytic processing. We have included a note in the manuscript to highlight the importance of performing preliminary tests to determine an adequate concentration of substrate for determining V_{max} values.

2. They showed the substrates with different amino acid substitutions gave different V_{max} . However, how does that correlate with the infection? Does the virus with unfavorable protease cleavage sequence infect the cells ineffectively? Could they provide the cell-based or animal studied information published in the literature to rationalize their substrate specificity data?

It has been shown that MERS-CoV S protein can be activated by a wide variety of proteases. It was previously demonstrated that MERS-CoV S could be proteolytically processed by cathepsins and TMPRSS proteases. We have shown that in addition to being cleaved by these proteases, MERS-CoV could be cleaved by furin at the S2' site ¹. This adds an extra "layer" of activation that allows the virus to gain a wider tropism, in particular unlocking access to cells expressing high levels of furin. Mutating the cleavage site to remove furin cleavability at S2' does not abrogate entry into host cells, however, it does decrease efficiency of entry ^{1,2}.

3. In Supplemental Figure 1A, I cannot tell which of the EMC/2012 mutS-I S2' and HKU205 S2' has higher activity.

The differences in furin proteolytic processing between MERS-CoV EMC/2012 mutS-I S2' and HKU205 S2' are very minor, especially considering the error bars associated with the measurements and that the Vmax values for both peptides were close to 0. We refer the reviewer to the supplemental table 2 showing the Vmax values for both peptides.

4. Why did they choose these particular cleavage sites if there are other furin protease cutting sites?

In a previous study, both the MERS-CoV S S1/S2 and S2' sites have been analyzed by fluorogenic peptide assays for furin cleavage ¹. In the example shown in the protocol, we want to present how mutations at cleavage sites impact proteolytic processing. S2' was chosen here because the cleavage site mutations found in camel-derived MERS-CoV S (HKU205 and mor213 strains) contain mutations at S2' ².

Reviewer #2:

Manuscript Summary:

The manuscript does a good job of briefly summarizing the roles for proteases in enveloped virus - cell entry. Accurate statements are provided. The methodologies to measure in vitro protease activities are clearly described and are useful.

Major Concerns:

1. All of the experiments use furin protease, however, the manuscript emphasizes that the methodologies are suitable for other proteases as well. Furin has a fairly well-documented cleavage substrate structure, while other virus-activating proteases (ie., cathepsins) have less defined or perhaps promiscuous cleavage sites. Therefore, the findings obtained from assays with cathepsins (or proteases other than furin) might be more informative. For these reasons, it might be useful to include cathepsins or other proteases in the visualized experiments.

Thank you for your suggestion of including other virus-activating proteases as examples in our manuscript. While we agree that it would be interesting to include proteases which have less-defined cleavage sites, such as cathepsins, we think for the purpose of clarity of the protocol to keep the focus on furin, which has a clearly defined cleavage site. This is especially important because we want to show examples of the sensitivity of the assay by including peptides that have single mutations that have a big impact on furin cleavability.

2. The in vitro cleavage assay may be amenable to the evaluation of specific protease inhibitors (ie., crude protease mixtures may cleave FRET substrates and the relevant cleaving proteases identified by introducing specific protease inhibitors. Does this work and if yes, the approach might be added into the manuscript.

We have previously performed experiments using this assay to test the effect of protease inhibitors. This has been performed by our lab with fluorogenic peptides derived from influenza HA ³. We have added this point in the discussion section to highlight this important point for our manuscript.

3. The results from the in vitro experiments can potentially be used to refine bioinformatic cleavage scoring algorithms. This might be mentioned, perhaps along with more information on whether the existing scoring algorithms have ever been invalidated by ongoing in vitro peptide cleavage assays of the

sort

used

here.

We agree with the reviewer and have added this remark to the manuscript.

4. The key limitation of the assay (peptides do not reflect native protein structures) is important and thoroughly described. This is good. What might be added are published observations that proteolytic cleavage sites are sometimes exposed on viral fusion proteins only after the fusion proteins have bound to cells or cell receptors. So the biologically important regulation of proteolytic cleavage is at the level of protein conformation. This might be emphasized a bit more. Also, it might be more explicitly stated that the in vitro peptide cleavage assays can generate false positive data (positive in vitro cleavage but no cleavage of native fusion protein) but are unlikely to generate false negative data. This is stated, but not as explicitly as some readers may desire (this latter issue is a minor point).

We agree with the reviewer and have added these remarks to the manuscript.

Minor Concerns:

None. The manuscript is well written.

Reviewer #3:

Manuscript Summary:

The manuscript by Jaimes et al. describes studies on a fluorogenic peptide cleavage assay to define proteolytic cleavage activity of viral protein cleavage site motifs. Viral protein cleavage is a critical aspect of initiation of infectivity for a number of important pathogens such as influenza and coronaviruses, and cleavage phenotype can be critical for host range and pathogenicity. The assays reported in this manuscript allow for rapid screening of proteases and the analysis of cleavage motifs that mimic the sites within viral glycoproteins. Although there are a few limitations, as addressed by the authors in the discussion section, this rapid screening serves as a foundation for more detailed studies that can be performed on expressed proteins or mutant viruses to gain a further understanding of this important topic. The experimental details are well described and the data presented are solid. The paper is concise and well written, and appears to be a good fit for JoVE.

Major Concerns:

None

Minor Concerns:

None

Reviewer #4:

Manuscript Summary:

In this manuscript, the authors describe a very interesting methodology to investigate the putative cleavage sites of peptides. The method is well described with sufficient details of the protocol to reproduce and adapt the experiments to various peptides and proteases. moreover, the authors describe

the applications of their methodology to putative cleavage peptide sites of spike proteins from coronaviruses.

Major Concerns:

The description of blank, positive and negative controls is missing in the protocol.

[We added a description to the protocol section of the manuscript.](#)

Minor Concerns:

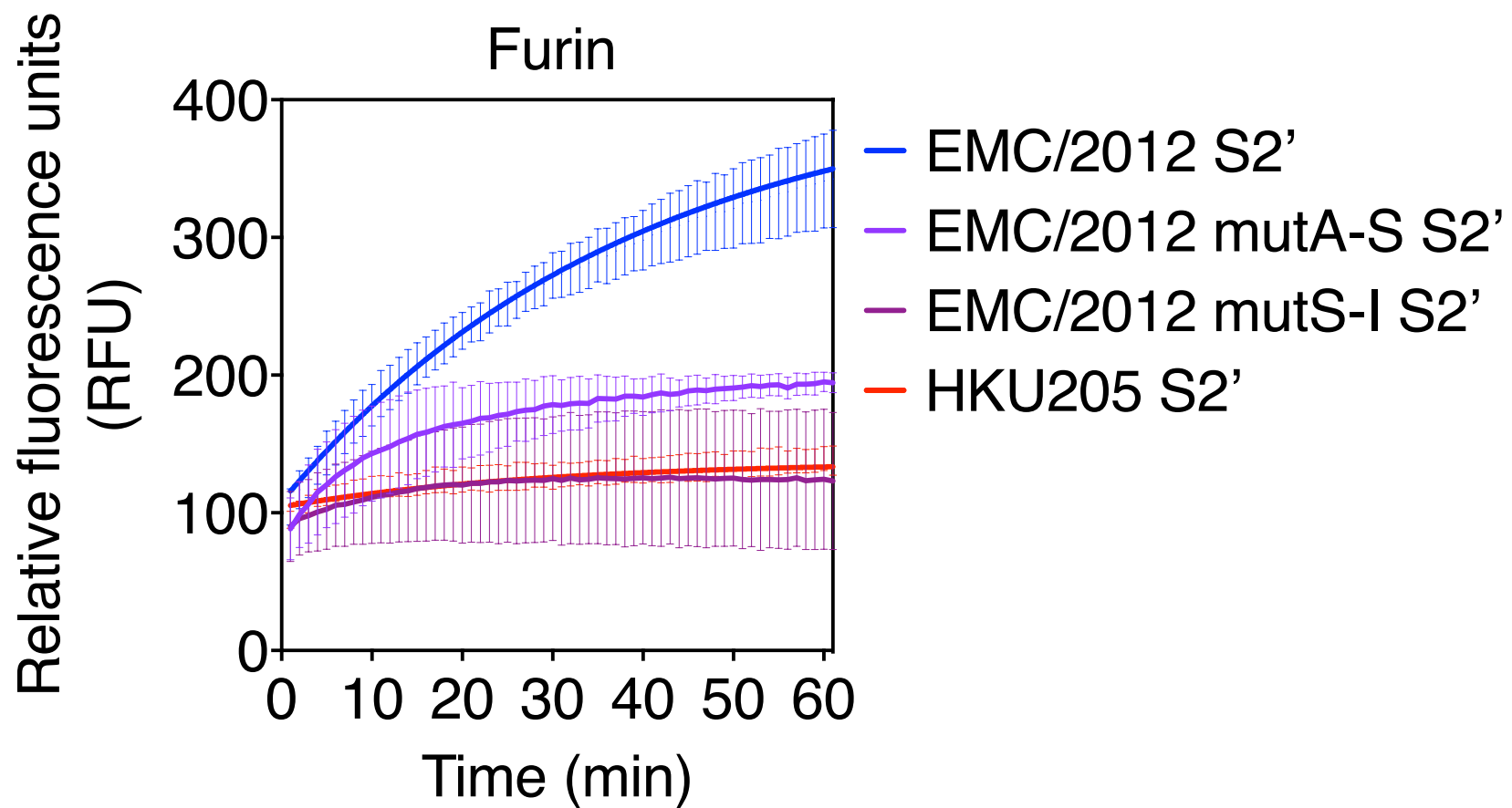
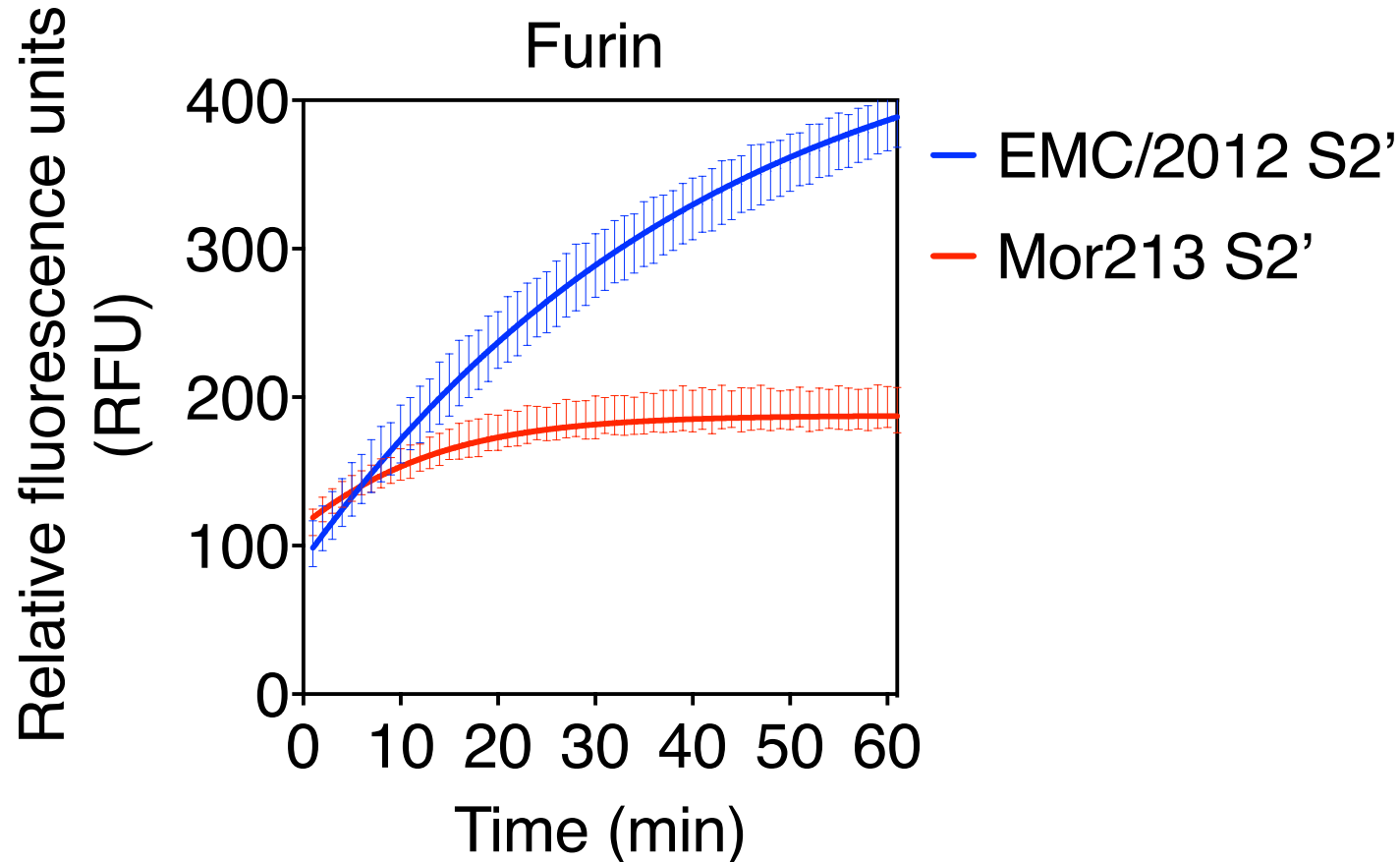
The authors should insist on the different applications of their methodology in the discussion section and should give more examples of applications beside the cleavage site of viral fusion peptides.

[We included the suggestion when addressing reviewer 2's major concern 3 and 4.](#)

References:

- 1 Millet, J. K. & Whittaker, G. R. Host cell entry of Middle East respiratory syndrome coronavirus after two-step, furin-mediated activation of the spike protein. *Proceedings of the National Academy of Sciences*. **111** (42), 15214–15219 (2014).
- 2 Millet, J. K. *et al.* A camel-derived MERS-CoV with a variant spike protein cleavage site and distinct fusion activation properties. *Emerging Microbes & Infections*. **5** (12), e126, doi:10.1038/emi.2016.125, (2016).
- 3 Hamilton, B. S. *et al.* Inhibition of influenza virus infection and hemagglutinin cleavage by the protease inhibitor HAI-2. *Biochemical and Biophysical Research Communications*. **450** (2), 1070-1075, doi:http://dx.doi.org/10.1016/j.bbrc.2014.06.109, (2014).

Peptide	Furin Vmax (RFU/min)
MERS-CoV EMC/2012 S2'	6.3 ± 1.2
MERS-CoV EMC/2012 mutA-S S2'	3.6 ± 1
MERS-CoV EMC/2012 mutS-1 S2'	1.1 ± 0.9
MERS-CoV HKU205 S2'	0.5 ± 0.1
MERS-CoV Mor213 S2'	1.0 ± 0.1
Peptide	Furin Vmax (RFU/min)
FECV I And-4 S1/S2	100.4 ± 7.6
FIPV I Black S1/S2	-1.4 ± 1.3
Peptide	Furin Vmax (RFU/min)
FECV I And-4 S2'	0.3 ± 0.1
FIPV I Black S2'	0.3 ± 0.1
FECV II 1683 S2'	5.5 ± 1.0
FIPV II 1146 S2'	-0.4 ± 0.1
Peptide	Trypsin Vmax (RFU/min)
FECV I And-4 S1/S2	413.3 ± 39.6
FECV I And-4 S2'	359.7 ± 22.1
FIPV I Black S1/S2	317.7 ± 25.3
FIPV I Black S2'	85.2 ± 5.9
FECV II 1683 S2'	342.3 ± 27.3
FIPV II 1146 S2'	152.2 ± 22.5

A**B**

Supplemental Figure 2

