Please find our responses to the editorial and peer-review comments below.

**Editorial comments:**  
Changes to be made by the Author(s) regarding the manuscript:  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The revised version of the manuscript has been proofread.  
2. Please revise the title to be more concise if possible.

We shortened the title.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: SpectraMax Gemini, Molecular Devices, SoftMax, Costar, Microsoft Excel, etc.

We removed all commercial language.  
4. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

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 Vmax of these peptide substrates. This reviewer has the following concerns or questions to be answered.

Major Concerns:

1. They have measured Vmax values of different fluorogenic peptides cleaved by Furin. However, by definition, Vmax is the maximal activity with a saturated amount of substrate (at least 5-fold of Km). From the description, they did not use different substrate concentrations (0.5-5-fold Km) to determine the Vmax. 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 Km and Vmax, so they can also discuss about the Km (substrate affinity). If they are sure 50 μM is high enough (please explain), they can claim what they measured are the Vmax.

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 Vmax values.

2. They showed the substrates with different amino acid substitutions gave different Vmax. 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 cleave 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 arrogate 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. moreove, the authors de scribe the applications of their methodology to putative cleavage peptide sites of sipke proteins form 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).