We greatly appreciate the feedback that was provided to us by these reviewers. We feel that our publication is stronger because they were willing to serve as reviewers. We have addressed the review concerns in red below.

Manuscript Summary:  
Authors describe a protocol to directly asses the effect of temperature on enzyme activity. This Isothermal Titration Calorimetry protocol is automated and generally applicable, since it is not based on a coupled reaction. It provides information complementary to measurements of protein unfolding. This protocol should of interest to the users of JoVE.  
  
Major Concerns:  
The ITC assay requires relatively large amount of enzyme, especially in comparison with coupled reaction based assays.

We have included the amount of enzyme required per reaction as a limitation of this assay.  
  
Minor Concerns:  
The details of the protocol are specific to one type of instrument.

We only have the TA ITC. We are not familiar enough with other instrumentation to include instructions for those.  
  
Reviewer #2:  
  
Manuscript Summary:  
The manuscript describes a protocol to determine the stability of enzyme activity over time using ITC. The method described is interesting and potentially relevant to evaluate the best conditions to maximize the enzyme usability over time. However, in my opinion some points need to be clarified.

Major Concerns:  
In the abstract the authors state that "This work demonstrates a new method for measuring the stability of enzyme activity by isothermal titration calorimetry (ITC)". I think that this protocol would be more robust if the authors provide a sort of "proof of concept" for this demonstration. In other words, it would be useful if they compare the results that obtained here with this method with data reported in the literature using a different technique. This would provide a clear demonstration of the reliability of their methodology, and would be more important if one considers that the decrease of activity over subsequent titrations could be due to product inhibition effects.

## In a previous publication, “Calorimetric Methods for Measuring Stability and Reusability of Membrane Immobilized Enzymes”, we compared a spectrophotometric enzyme activity assay to the ITC method using invertase. We have referenced this paper and the comparison. There are also several other papers that use other methods to measure enzyme stability. We did not do additional experiments because of time constraints, but we do think that there is sufficient information in the literature for interested parties to determine if this would be a better method relative to more conventional methods.

## Indeed, the authors state that "the product concentrations were much lower than the inhibition constant for the product", but they do not provide any reference for this statement. How do they know that under the conditions used there is no product inhibition effect? As this is a critical point, I think that this should be demonstrated with a dedicated experiment, for example performing the experiment at the different time points without any previous substrate titration (i.e. with no product present in solution).

We addressed this by performing the experiment in the presence of 54 mM glucose and galactose, which is the highest concentration that the glucose and galactose get during the course of our experiment with lactase. We did see that there was some product inhibition (a 27% decrease in the peak heat rate). However, in our assay we see a 78% reduction from the first to the last injection. We could do the same assay, but with less substrate per injection and decrease the product concentration.  
  
Minor Concerns:  
- Ref 12 is outdated. There are more recent reviews that report how to measure enzymatic activity with ITC, one also published in JoVE (<https://www.ncbi.nlm.nih.gov/pubmed/24747990>).

We have updated the reference.

- Figure 6 is not clear to me: in the text it is written that "the peak heights decrease linearly with time indicating decreasing enzyme activity" but I do not see time on the x axis, while enzyme concentration is reported.

We have edited the figure legend to better match the figure.

- Just out of curiosity: why a so large amount of enzyme (10 mL) and substrate (50 mL) solutions are prepared for the experiments? As the instrument requites small volumes of solutions, these amounts would seem a waste of material.]

We run many enzyme assays and have found that when we make up large volumes it cuts down on the amount of error in our measurement. The enzymes that we have used thus far have been relatively inexpencive so making up It is not required to be this way.

- There are some grammatical errors through the manuscript, so I would recommend copy editing. Other mistakes through the text:

We have addressed these grammatical errors.

o Abstract, line 26 "rate of loss of enzyme activity" does not feel right to me. I would change with "loss of enzyme activity over time".  
o Page 3, line 170 "Both enzymes were run…". Enzymes are not run, so I guess the authors meant that "both enzymatic reactions were run".  
o Page 4, line 183 "peak height of the endothermic reaction": I think the authors meant "exothermic".

We did mean to say endothermic as lactose hydrolysis endothermic reaction.  
o Page 7, line 330, I think that "Figure 4" is "Figure 5"  
o Page 8, line 335, I guess that "Figure 5" is "Figure 6".