LOMA LINDA UNIVERSITY
School of Medicine

October 7, 2015

Dr. Nam Nguyen,
Science Editor, and
Aleksandra Jachtorowicz
Deputy Director of Editorial - Life Sciences
Journal of Visualized Experiments
1 Alewife Center, Suite 200
Cambridge, MA 02140

Dear Drs Nguyen and Jachtorowicz;

Thank you for the editorial comments and we are especially grateful for the reviewers insight and suggestions. The manuscript will be better because of their input.
Please see the attached responses to each editorial concern and each reviewer concern and comment.

Also note that all of the authors have reviewed the revised manuscript, figures and comments.

Also, congratulations on receiving your first impact factor. Your hard work is paying off and I am sure that the impact will continue to climb in this highly interactive environment.

Sincerely,

Kerby C. Oberg, MD, PhD
Senior Administrative Professor
Department of Pathology and Human Anatomy,
Divisions of Human Anatomy and Pediatric Pathology

Responses to Editor and Reviewer comments/critiques:

1. Please proofread the manuscript for spelling or grammar issues:
We have thoroughly reviewed the manuscript for spelling and grammar issues and made corresponding corrections.

2. Please include an ethics statement indicating that the protocol follows the animal care guidelines of your institution.
We have added an ethics statement as requested – lines 108 -115

3. Please the protocol text is a hard 2.75 page limit of highlighted protocol text for filming.
The highlighted protocol has been reevaluated and reduced as requested.

4. Some additional details are required:
•5.2.1 - What steps are involved with this process? Any tips?
•5.2.3 - What magnification should be used?
The additional details have been addressed in the protocol. Tips see 5.2.2.1 lines 355-357. Magnification for different steps see lines 343-344, 349-350, and 352

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
In this paper, the Authors describe a rapid, effective and cheap system for validating regulatory regions, based on transfection of chicken embryos with reporter plasmids. This system is particularly useful to study developmentally-regulated enhancers.
Thank you

Major Concerns:
1. The protocol is well described, but probably not easy to understand for a lab where there is no expert in chicken development. The video associated with the manuscript will be essential to acquire these techniques.
We have carefully evaluated the protocol with this concern in mind and have revised accordingly while keeping within the 2.75 page limit.

2. Considering the inter-laboratory variability, should each lab set up the best electroporation conditions (voltage, pulses)? The Authors should comment on the setting up of the electroporation conditions.
Appreciate the comment. We added our approach to optimizing the EP parameters for each embryonic stage or condition — see lines 463-465

Minor Concerns:
1. It would be useful to mention if the plasmids are available (Addgene, which laboratories can provide them).
The p*tk*EGFP plasmid was a gift from M. Uchikawa and H. Kondoh. Addgene does not have tk-EGFP but they have variants (tk-RED) and they refer to other companies that offer additional variant reporters (eg., Thermo Scientific: luciferase). A note was added to the acknowledgement section and a comment in the reagent excel file.

2. The cloning step is a standard procedure, the list of reagents for this step may be excluded from the text (besides the endofree maxiprep that is essential, as mentioned in the discussion, to reduce the embryo damages).
The common molecular biology reagents (enzymes, etc) have been deleted from the materials list in the excel file as requested, except for the endotoxin free maxiprep kit which is an essential element of the protocol. Mineral oil for the oil bubble technique was also added to the materials list.

Additional Comments to Authors:
N/A

Reviewer #2:
Manuscript Summary:
Given the emerging high-throughput methods for enhancer discovery, it will be useful to identify techniques for rapid validation of enhancer elements. The authors describe rapid chick bioassays for in vivo validation of putative regulatory elements. They discuss whole animal and targeted regional electroporation methods of introducing putative regulatory elements which are tested for their ability to drive reporter expression during chick development. The method is well-written, and the video promises to be informative. The authors have done a very nice job, and I have only a handful of suggestions for improvements:
Thank you

Major Concerns:
It would be good to suggest controls -- other than mutated enhancers shown in your representative data -- that might be easier to construct. Also, you mention the RFP construct positive control in line 424, but it might be good to mention this up front. I would add discussion of both negative and positive controls in 1).
In 1.4 of the protocol we indicate that the empty p*tk*-EGFP expression is used as a negative control to demonstrate basal activity of the plasmid and the beta-actin-driven RFP is used as a positive control and indicator of transfection efficiency. (Lines 127-129)

Minor Concerns:
Would it be appropriate to include an ethics statement and an indication that those who use this method should seek vertebrate animal approval?
An ethics statement regarding the use of vertebrate animals and the need to inquire about whether approval is needed for embryos has been included. Lines 108-115

Are there any safety precautions to be taken for the electroporation procedure?
Thanks for noting this – we have added a precautionary comment regarding the use of the electroporator. See step 2.3.4.1 in the protocol lines 218 - 221

Is a dissecting scope used/helpful during the embryo preparation?
Added "under dissecting microscope" when used throughout the protocol – see lines 197-198, 253 and 352.

You may wish to consider demonstrating the oil bubble method (line 419) in your video protocol, as it might be useful to see this step. It looks tricky.
The oil bubble was included in the video protocol – see lines 207-210 and lines 307-309

Line 102: TO validate
Thank you, the "to" was added in front of the validate in line 104

Line 389: ptk should be italicized; kinase instead of kiniase
Thank you, these have been corrected – see line 429 and 445

Line 447: maintain instead of maintaining
Thank you this has been corrected – see line 504

Fig. 1D: What is the pink area in the X-sectional view?
The pink area is illustrating a somite in x-section and extraneous to the lateral plate being electroporated. I have removed the color to minimize it distracting readers from the point of the illustration. I have also included an alternate image that labels the somite (so) and neural tube (nt) to improve clarity of the structures illustrated, but I think these are not necessary if the color has been removed. I prefer the first image - Fig 1mA.tif. If the alternate is preferred we will need to modify the legend to annotate these additional terms.

Fig. 2 E, M and N: These printed pretty dark, so you might want to brighten these images.
As requested, we have brightened images M &N (and also K & L within the same panel). We have also brightened E also with a slight increase in contrast.