Response to Reviewers

JoVE Submission JoVE58907

We outline our point-by-point response to the editor 2 reviewers comments below. To aid in review of these revisions, we have included revised manuscript text in this document. Our comments are in bold.

Editorial Comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

**Done. We have made several small changes to aid in readability.**

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

**Figure 6 is a modified version of a figure initially published by our group in:**

**"Amino acid-level signal-to-noise analysis of incidentally identified variants in genes**

**associated with long QT syndrome during pediatric whole exome sequencing reflects**

**background genetic noise.” Heart Rhythm 2018 Mar 2. pii: S1547-5271(18)30142-5. doi:**

**10.1016/j.hrthm.2018.02.031**

**Elsevier journal authors retain the right to use or re-use excerpts (including figures) in other works with proper acknowledgements. We have provided this acknowledgement in the figure legend. For the convenience of the editor, we have also attached the written communication with the Elsevier editorial desk and the appropriate policy.**

3. Please provide an email address for each author.

**Added**

4. Please refrain from using indentations.

**Removed**

5. 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: EXCEL, GSL Biotech LCC, etc.

**Removed and the Table of Materials referenced and updated.**

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

**Removed.**

7. 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.

**We have extensively revised the protocol to achieve this. While we have significantly reduced the number of “Notes” that are included in the protocol portion of the manuscript, the ones that remain we feel are critical for usability of the protocol.**

8. 2.1, 2.2, 2.3.4, 2.4.1, 2.4.2, 2.6, 3.1, 3.2, etc.: Please write the text in the imperative tense.

**As above.**

9. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

**As above. We have moved topics on MAF threshold and rolling average to the Discussion.**

10. 5.1.3 and sub-steps: Please describe the specific actions being performed here. If there are no actions, they should be removed from the protocol.

**Revised.**

11. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Software must have a GUI (graphical user interface) and software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.) 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.

**Done. We have added more functional details. Due to the revision that requires us to remove “Excel” from the manuscript (referencing the Table of Materials) this limits our ability to describe how to use this software if we must keep the software a generic description.**

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

**Done**

13. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

**Done**

14. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

**Done**

15. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

**Done**

16. References: Please do not abbreviate journal titles.

**Done**

Reviewer #1:

1. Point 4.3 Rolling average - further guidance could be provided as to what this should be - e.g. should it be 5? and what might this be based upon.

**Thank you for pointing out our lack of clarity. It’s true that we do not specify the range of this rolling average beyond the given example, nor do we provide guidance on factors that may explain (1) why 5 was chosen and (2) when a bigger or smaller range may be appropriate. +/- 5 was utilized in KCNQ1 given the size of the transmembrane domains (~10 amino acids) and needing to illustrate a signal to noise “resolution” that would be less than these domains. The rolling average can similarly be adjusted to suit 1) protein domain size, 2) length of the primary sequence, 3) the prevalence of rare variants found in healthy individuals, 4) the signal-to-noise graph itself. We address this with the following addition to the Discussion and have included relevant addition below.**

**“Setting a rolling average for MAFs allow for inference of involvement to neighboring amino acids. For example, if amino acid position 35 contains a pathologic variant and resides in a critical protein domain, then position 36 may have a degree of pathogenicity when mutated. Likewise, should a stretch of primary sequence have a large amount of rare control variants, then amino acids within this region that do not host rare variants may yet have a higher likelihood of containing rare variants found in a population. While the rolling average in this protocol is +/- 5, this range can be vary based on the user’s desired level of resolution of signal-to-noise ratio and the specific protein being studied. In the example of LQTS, the interrogated *KCNQ1*-encoded KCNQ1 channel has several transmembrane domains spanning ~10 amino acids, prompting the authors to adjust their desired resolution to reflect significant findings on that scale1. For proteins with a longer primary sequence and protein length, the span of the rolling average may need to be increased due to larger spans of protein sequence without control variation.”**

Reviewer #2:

1. Introduction - as this protocol uses long QT syndrome as the primary example, the authors ought to cite some of the sentinel work pertaining to background genetic noise this protocol expands upon (Ackerman, Mayo Clinic Proceedings, 2003, Kapa et al, Circulation, 2009, etc).

**Thank you for pointing out this oversight, as these works contributed heavily to our current work. Citations for these papers have been added. The introduction now states:**

**While a number of genes have been linked to the development of this disease, mutations in *KCNQ1-*encodedIKs potassium channel(KCNQ1, Kv7.1) is the cause of LQTS type 1 and is utilized as an example below2. Illustrating the complexity in variant interpretation, the presence of rare variants in LQTS-associated genes, so called “background genetic variation” has been previously described3,4.**

1. In light of the recent work by Bagnall et al, JACC, 2018 illustrating an expanded role of pathogenic deep intronic splicing variants in the pathogenesis of HCM, the authors ought to consider alerting readers that this protocol, as written, would miss such variants. That said, expansion of this approach to a genome-wide approach may help identify intronic/intergenic "hotspots" that underlie monogenic disease.
2. The MAF cutoffs proposed in this protocol are reasonable for the assessment of highly penetrant monogenic-disease causative variants. However, we are slowly learning that the genetic architecture of most cardiomyopathies and cardiac channelopathies, in many cases, is much more complex than the current monogenic/single gene model. As such, I would urge the authors to consider adding a sentence or two to the discussion that highlights for readers that so-called "risk alleles", variants with MAFs in gnomAD far greater than disease prevalence, will not be assessed by this approach. However, these variants may still contribute to cardiovascular diseases we still consider to be monogenic [this is illustrated in the channelopathy realm by p.Asp85Asn-KCNE1 (LQT5; Giudicessi et al, Circulation, 2018 and Lane et al, Heart Rhythm, 2018) and in the cardiomyopathies by p.Val122Ile-TTR/p.Val142Ile-TTR (Familial Amyloid Cardiomyopathy; Buxbaum and Ruberg, Genet Med, 2017) and p.del25-MYBPC3 (HCM; Dhandapan et al, Nat Genet, 2009)]. It is important to alert readers that the approach/MAF cut-offs outlined in this protocol will not assess these relatively common, but potentially clinically important variants.

**Both excellent points. Our protocol’s signal-to-noise resolution is limited to exonic changes that are represented by amino acid changes, which largely omits intronic genetic variants. We now address this in the discussion as a method limitation, with reference to Bagnall et al’s work. Further, we address the reviewers concern that our protocol may also exclude common potentially pathogenic variants by way of a MAF threshold, and note this as well as a limitation.**

**“As detailed, this protocol focuses specifically on amino-acid level changes resulting from exonic gene variants that code for amino acids, which excludes the role that pathogenic intronic splicing variants may play in monogenic disease. Given their recently demonstrated role in cardiomyopathies, expansion of the resolution this approach may be warranted to identify intergenic “hotspots” as well.** **Furthermore, the application of a MAF threshold may miss certain “risk alleles” that, though existing in the population with a MAF higher than that of disease prevalence, may contribute to disease pathogenesis5,6. Despite these limitations, this analysis is adaptable and can play a key role in providing clinicians a relative probability of disease pathogenicity when appropriate applied.”**

**Below are the citations for the above text changes. Please note that the numbering does not reflect the order of citations in the revised manuscript**

**1 Landstrom, A. P. *et al.* Amino acid-level signal-to-noise analysis of incidentally identified variants in genes associated with long QT syndrome during pediatric whole exome sequencing reflects background genetic noise. *Heart Rhythm.* 15 (7), 1042-1050, (2018).**

**2 Wang, Q. *et al.* Positional cloning of a novel potassium channel gene: KVLQT1 mutations cause cardiac arrhythmias. *Nat Genet.* 12 (1), 17-23, (1996).**

**3 Kapa, S. *et al.* Genetic testing for long-QT syndrome: distinguishing pathogenic mutations from benign variants. *Circulation.* 120 (18), 1752-1760, (2009).**

**4 Ackerman, M. J. *et al.* Ethnic differences in cardiac potassium channel variants: implications for genetic susceptibility to sudden cardiac death and genetic testing for congenital long QT syndrome. *Mayo Clin Proc.* 78 (12), 1479-1487, (2003).**

**5 Bagnall, R. D. *et al.* Whole Genome Sequencing Improves Outcomes of Genetic Testing in Patients With Hypertrophic Cardiomyopathy. *J Am Coll Cardiol.* 72 (4), 419-429, (2018).**

**6 Giudicessi, J. R., Roden, D. M., Wilde, A. A. M. & Ackerman, M. J. Classification and Reporting of Potentially Proarrhythmic Common Genetic Variation in Long QT Syndrome Genetic Testing. *Circulation.* 137 (6), 619-630, (2018).**