**Response to Reviewers**

**JoVE 50710**

**Won et al., Detecting Somatic Genetic Alterations in Tumor Specimens by Exon Capture and Massively Parallel Sequencing**

**Editorial comments:**

Protocol text: Please highlight less than 3 pages of text to identify which portions of the protocol are most important to film; i.e. which steps should be visualized to best supplement the written section of the protocol.

*We have reduced the highlighted section to less than 3 pages of text, as requested.*

Please remove all trademark symbols (ie uploaded Materials table).

*We have removed all trademark symbols, as requested.*

Please use the attached version of your manuscript for future revisions.

*We have made several revisions, with changes tracked, to the version of the manuscript supplied with the reviewers’ comments. The specific revisions are discussed below in our point-by-point response to the reviewers.*

**Reviewer #1:**

A number of software tools were also listed in Data Analysis method section. However, much more detailed description of the implementation and integration of different analysis methods would be very beneficial.

*We have significantly modified Section 9 (“Data Analysis”), adding considerable details for each step in our analysis pipeline. Specific parameters and filtering criteria are listed for the algorithms that we use for adapter trimming, mutation calling, and indel calling. Additional details are provided for our process for determining copy number alterations.*

More detailed description about the capture library design would be much appreciated, especially for the capture of translocations.

*We have created a new table (Table 1 in the revised manuscript) describing the overall features of our custom capture design. In summary, our panel contains all protein coding exons of 279 cancer-associated genes (4,535 total exons). We also include 14 introns from 7 genes that are recurrently rearranged in solid tumors, as well as >1000 SNPs for fingerprint genotyping and quality control. The total target territory is approximately 880 kilobases of genomic sequence.*

Basic technical performance was presented. But more in-depth analysis of the platform would be important to evaluate the platform for potential clinical applications. The analysis could address coverage and uniformity of sequencing as well as capture and sequencing bias. The variant analysis could address variant detection sensitivity, specificity and reproducibility for different classes of variants, including single nucleotide variants, small indels, large indels, translocations and copy number changes.

*We have added a new paragraph to the Discussion addressing these points. Please see the revised manuscript for the full paragraph. In summary, we stress the following points: (1) A benefit of the targeted approach is that it is feasible to optimize capture probes such that all exons are covered uniformly and sufficiently for mutation detection. (2) Following iterative improvements to our design, a typical sequencing experiment produces no more than 2% of exons at less than 20% of the median coverage for the entire sample. Thus, for a tumor sequenced to 500x sequence coverage (a conservative estimate as displayed in Figure 1), >98% of exons will be covered by >100x depth, guaranteeing high sensitivity for identifying low frequency mutations. (3) By also capturing select introns of recurrently rearranged genes, we are able to identify structural rearrangements producing fusion genes even when only one fusion partner is captured.*

**Reviewer #2:**

Targeted sequencing is a well-known, well-used method. This article does not illuminate anything new or little-known in the method. The video would involve mostly footage of workers pipetting.

*We agree that experimentalists familiar with targeted sequencing methods may not gain as much from this video and accompanying protocol as someone who is new to next-generation sequencing. However, we believe that a detailed exposition of our method, especially in its simplicity, will be of significant value to laboratories beginning to invest resources and personnel into next-generation sequencing workflows. Our experience is that labs that have traditionally relied on specialized core facilities to handle all of their sample prep and sequencing are now beginning to prepare their own samples given the wider accessibility of sequencing platforms and options. We hope that with the availability of protocols like ours, this process will be less intimidating for labs new to the field. Further, it is our understanding that the Journal of Visualized Experiments has not yet published a protocol or produced a video describing the preparation of libraries for targeted sequencing, and we believe that our method will serve as a valuable resource.*

The area of great interest, the actual analysis of the data, is only touched on in a manner that it would be impossible to re-capitulate the methods in another lab.

*This sentiment was shared by Reviewer #1. As described above, we have significantly modified Section 9 (“Data Analysis”), adding considerable details for each step in our analysis pipeline. Specific parameters and filtering criteria are listed for the algorithms that we use for adapter trimming, mutation calling, and indel calling. Additional details are provided for our process for determining copy number alterations.*

**Reviewer #3:**

If the authors have a preference for which custom capture probes to use, i.e. Agilent versus Roche versus IDT, might be worth mentioning or discussing further.

*As mentioned in our response to Reviewer #1, we have added a new paragraph to the Discussion describing our capture performance and the uniformity of sequence coverage we have achieved through iterative improvements to our probe design. In the revised text, we state that the uniformity of sequence coverage can be largely attributed to the flexibility of the Nimblegen SeqCap system, in which probes are synthesized to different lengths in regions of different nucleotide composition so that they may have similar melting temperatures. We also describe how we have successfully spiked in individually synthesized biotinylated oligonucleotides from IDT to add new content to our panel and to boost the coverage of regions that are difficult to capture and/or sequence.*