Dear Dr. Upponi,

Re: JoVE54090R3 "Targeted RNA Sequencing Assay to Characterize Gene Expression and Genomic Alterations"

Please find attached a revised manuscript and reply for reviewer comments.

We appreciate the reviewer comments and suggestions, and hope that we have clarified questions with our revisions and item by item reply.

We look forward to hearing from you,

Best,

Sameek

CC: [nandita.singh@jove.com](mailto:nandita.singh@jove.com)  
  
Dear Dr. Roychowdhury,  
  
Your manuscript JoVE54090R3 "Targeted RNA Sequencing Assay to Characterize Gene Expression and Genomic Alterations" has been peer-reviewed and the following comments need to be addressed.   
  
Please keep JoVE's formatting requirements and the editorial comments from previous revisions in mind as you revise the manuscript to address peer review comments. Please maintain these overall manuscript changes, *e.g.*, if formatting or other changes were made, commercial language was removed, *etc.*   
  
Please track the changes in your word processor (*e.g.*, Microsoft Word) or change the text color to identify all of the manuscript edits. When you have revised your submission, please also upload a separate document listing all of changes that address each of the editorial and peer review comments individually with the revised manuscript. Please provide either (1) a description of how the comment was addressed within the manuscript or (2) a rebuttal describing why the comment was not addressed if you feel it was incorrect or out of the scope of this work for publication in JoVE.  
  
**Your revision is due by Dec 16, 2015. Please note that due to the high volume of JoVE submissions, failure to meet this deadline will result in publication delays.**  
  
To submit a revision, go to the [JoVE Submission Site](https://urldefense.proofpoint.com/v2/url?u=http-3A__www.editorialmanager.com_jove&d=BQMGaQ&c=k9MF1d71ITtkuJx-PdWme51dKbmfPEvxwt8SFEkBfs4&r=MtcULrh0sE9nBgE6zAg-wAp25Dmb6hzoRykJenXK1pQ&m=fZPnup4wwvX68I_yAAaeZOrggHF66fi2iDVyVER7Ob4&s=2d3HovaEqAjIYn0DNta6DtWfqd_qS7pm_tSaEvxGOAE&e=" \t "_blank) and log in as an author. You will find your submission under the heading 'Submission Needing Revision'.  
  
Sincerely,  
  
Jaydev Upponi, Ph.D.   
Science Editor   
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**Editorial comments:**  
***NOTE: Please download this version of the Microsoft word document (File name: 54090\_R2\_100715) for any subsequent changes.***  
  
•Please keep the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.   
  
•Formatting:  
-3.2.3, 5.4.4 – Please split into two steps.  
-3.2.9 – Please make the last sentence a note.  
-All figure legends should have a title and a brief description describing the figure.  
  
•Grammar:  
-Please copyedit the manuscript for numerous grammatical errors. In particular, many protocol steps contain sentences with comma splices or run-on sentences.  
-2.1.2 – “pre-hit” should be “pre-heat”  
-5.2.5 – “table top mini centrifuge tubes” should be “table top mini centrifuge”  
-Informal abbreviations should not be used in the manuscript. Please use “hybridization” rather than “hybe” throughout the manuscript.  
  
•Unnecessary branding should be removed:  
-5.5 – Illumina MiSeq  
-6.1 – FastQC – The name of a program should not be a section heading.   
  
•If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as “Re-print with permission from (reference#)” or “Modified from..” etc. And please send a copy of the re-print permission for JoVE’s record keeping purposes.  
  
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***NOTE: Please copyedit the entire manuscript for any grammatical errors you may find. This editing should be performed by a native English speaker (or professional copyediting services) and is essential for clarity of the protocol. Please thoroughly review the language and grammar of your article text prior to resubmission. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.***   
  
***NOTE: Please include a line-by-line response letter to the editorial and reviewer comments along with the resubmission.***   
  
  
**Reviewers' comments:**  
  
**Reviewer #1:**   
*Manuscript Summary:*  
This manuscript by Martin et al, "Targeted RNA Sequencing Assay to Characterize Gene Expression and Genomic Alterations", describes the methodology for performing capture RNA-Seq of select transcripts to derive a targeted gene expression profile and to reveal alterations such as sequence variants and gene fusions. It is well written and provides detailed experimental methods of the RNA/cDNA preparation steps. The manuscript also provides a nice representation of various QC metrics (gene expression levels, mapping statistics, alignment rates, insert sizes) and discussion of different types of alterations that can be detected in cancer samples. There are several issues detailed below, however, that would need to be addressed prior to publication.  
  
*Major Concerns:*  
1. In the introduction, the concept and rationale for targeted capture method are described, but it would be more informative to specify the capture parameters in detail. Which genes (and how many) were captured? How were the genes selected? Do probes include UTR sequence or just coding exons? How were the custom probes designed, and where were they ordered from? The source of capture probes should be listed either in methods section 5 (Hybridization, Capture, and Sequencing) or in the Table of Materials/Equipment.

**We selected exons for genes of interest in drug development including kinases, genes involved in common rearrangements such as transcription factors, and house keeping genes. Please see revised text on page 12.**

**Editor: Probes may vary based on commercial provider. Several commercial providers such as Agilent, Nimblegen, and IDT DNA Technologies can provide that are designed according to their best practices. We have followed the same format as in JOVE article Won et al., Detecting Somatic Genetic Alterations in Tumor Specimens by Exon Capture and Massively Parallel Sequencing.**

2. There is not nearly enough detail for the computational methods (Section 6: Data Analysis). The run parameters for every algorithm should be listed. For FastQC and RNA-seq QC, what are the desired or expected outputs, and what actions are taken based on different QC values? More detail is needed for the specific post-processing steps in 6.3.2. What thresholds or parameters are used for variant calling in 6.4?

**Editor: For analysis methods, please revised text on page 10. We have provided the same level of detail as seen in JOVE article Won et al., Detecting Somatic Genetic Alterations in Tumor Specimens by Exon Capture and Massively Parallel Sequencing. More than this would amount to 10+ pages of text.**

3. The paper states that the protocol is compatible with not only cell line RNA, but with frozen and FFPE RNA (Pg 4, lines 99-102). However, the results only show data from cell lines. As RNA from degraded materials have much lower RIN scores and shorter fragments thus resulting in lower quality cDNA and library yields for sequencing, are there modifications to the steps in the kit used here (TruSeq Stranded Total RNA kit) when starting with degraded RNA from FFPE tissue, besides increasing the input amount? Comparable results (especially QC metrics) should be shown using frozen and FFPE RNA to truly show the utility of this method in various sample types.

**Editor: RNAseq and capture has been applied to cell line and FFPE RNAs. For the purpose of the JOVE video and demonstration article, we have shown an example using cell line RNAs. There are at least five commercial entities with protocols that can be applied for cell line, frozen, or FFPE RNA. Demonstrating all possible variations and applications is outside the scope, but this provides starting point for potential directions for a reader.**

4. The data in Table 1 (Fusion Detection for Capture versus RNAseq) are perplexing. For ChimeraScan and TRIP, the number of fusion supporting reads is greater for unselected rather than captured RNA-Seq. This is in direct contrast to Tophat Fusion. Is this a mistake, or is there an explanation? This is not consistent with statement on p.12: "Generally, capture outperformed RNAseq in fusion detection with the presence of more fusion supporting reads." Furthermore, certain fusion events were missed by ChimeraScan and TRUP entirely; is there an explanation for this?  
**Thank you for pointing out this difference. The Table shows normalized fusion supporting reads for three different fusion tools. In some instances, one fusion tool may be more efficient than another at detecting different types of fusions. For this particular application, our preferred tool is Tophat fusion, however for the sake of demonstration, we have shown two other examples, and they do performly differently.**

**on p.12: "Generally, capture outperformed RNAseq in fusion detection with the presence of more fusion supporting reads." We have revised page 12, line 479 as follows:**

**“Capture RNAseq was successful in detecting fusions for all four cell lines.”**

**Editor: Differences in fusion tools is outside of the scope of this JOVE manuscript.**

*Minor Concerns:*  
1. In Figure 3, it would be helpful to also show the percent of reads mapping to target genes versus the rest of the transcriptome.  
**Thank you for this suggestion. Please see Figure 2B shows percentage of reads mapping to targeted region in Capture versus RNA seq in four cancer cell lines.**

2. In the Representative Results section, the authors describe the four cancer cell lines with known mutations. It would be helpful to clarify the types of known mutations (e.g., SNVs, gene fusions, etc).  
**Thank you for this suggestion, we have described the four cancer cell lines on page 11-12 in the Representative Results section with information detailing these cell lines as follows:**

**“Four cancer cell lines with known mutations were used to demonstrate the effectiveness of the RNAseq Capture technique (K562 with *ABL1* fusion, LC2 with *RET* fusion, EOL1 with *PDGFRalpha* fusion and RT-4 with *FGFR3* fusion).”**

3. It is nice to see the low % of rRNA sequences using the capture method. However, when using the capture method, is rRNA depletion really necessary? Looking at the % of rRNA sequences in rRNA depleted capture RNAseq vs traditional RNAseq doesn't accurately evaluate the efficiency of capture RNAseq in reducing rRNA sequences. The fair comparison of the efficiency of rRNA depletion would be to compare the % of rRNA in samples with rRNA depletion vs without rRNA depletion using the capture method in both.

**Thank you for this comment. Our demonstration shows rRNA depleted samples with or without enrichment. This experiment does not seek to or adequately compare the ribosomal depletion vs no ribosomal depletion steps.**

**Editor: The goal of this methods paper was to show how target enrichment can be utilized for targeted RNAseq. There are multiple ways to avoid sequencing rRNA, including depleting rRNA or enriching for other transcripts such as PolyA or probe hybridization. This is outside of the scope of this JOVE manuscript.**

*Additional Comments to Authors:*  
N/A  
  
  
**Reviewer #2:**   
*Manuscript Summary:*   
In this paper, Martin et al described a targeted RNA-seq method (Capture RNAseq) that focused on selected RNA products. They demonstrated the application of this method in four different cell lines using desktop sequencer followed by computational analyses to characterize gene expression, gene fusion and SNV. However, several questions need be addressed before the reader can fully appreciate the advantage and novelty of this method.  
  
*Major Concerns:*  
1. What is the difference/improvement of this method compared to the other targeted RNA-seq methods such as the ones described by Cieslik et al (2015) and Clark et al (2015)? The authors put a lot of effort into comparing Capture RNAseq with RNAseq, which has been thoroughly discussed by previous studies and shadowed the true technical advantage of this method.

**Editor: This is correct. This is not different or an improvement but rather a detailed description and video demonstration of targeted RNAseq. We have demonstrated how to complete this method.**

2. The application of targeted RNA-seq mainly benefits from its high sensitivity and accuracy, which is not well demonstrated in this paper. How to validate the gene expression levels detected by Capture RNAseq? Maybe the authors can use qPCRs to assess the targeted gene expression and see if that correlate with the results of Capture RNAseq and ordinary RNAseq. Additionally, internal controls such RNA spike-in (e.g. ERCC spike-in) or intergeneric DNAs can be helpful to estimate the relative expression levels. Furthermore, technical replicates should be included to show the reproducibility of the method.

**Editor: Recent publications have demonstrated sensitivity for gene expression and reproducibility using ERCC spike-in (example Clark et al 2015 Nat Methods). We do not think it is necessary to replicate these papers. This is outside of the scope of providing a detailed methods description and video demonstration of targeted RNAseq.**

*Minor Concerns:*  
1. Fig. 2A, there are quite some non-targeted genes which can also be detected as "highly expressed" in Capture RNAseq compared to RNAseq. Non-specific amplification/hybridization? Please explain.

**Editor: The reviewer’s comment is correct that these are most likely due to “non-specific amplification/hybridization”, and represent a very small percentage of transcripts assessed.**

2. Fig. 3F, in some samples (e.g. LC2), the rRNA percentage in rRNA-depleted RNAseq seems higher than usually reported.

**Editor: The reviewer’s comment is correct, LC2 sample has higher than normal rRNA %. These four samples represent real data, and in practice some samples may have variable rRNA depletion or target enrichment. In this instance, the preparation was successful in detecting the RET fusion gene and hence was not re-prepped. Protocols from manufacturers provide similar guidance.**

3. The authors claimed that Capture RNAseq "can be completed on desktop sequencers with rapid turnaround time compared to traditional RNAseq", however, data of comparison was now shown in the paper.  
**Thank you for this comment. While traditional rNAseq can be completed on HiSeq instruments or similar instruments, the number of individual samples needed per run to fill a run is significantly greater (24-48 samples per lane). This could thereby limit turnaround time.**

**We have revised text on page 3 as follows:**

**“RNAseq Capture has intermediate throughput, greater dynamic range and sensitivity, and is scaled for fast turnaround on desktop sequencers.”**

4. In PROTOCOL section, part 5. Hybridization, Capture and Sequencing, how were the probes designed? In part 5.2 Bead Preparation and Capture, line 318, what are the Wash Buffers I, II, III and Stringent? Were they from commercially available kits? Please specify.

**Editor: Probes can be designed with one of three major commercial supplies: Agilent, Nimblegen, IDT DNA Technologies. Wash buffers sources were removed as per JOVE instructions.**

5. There seems to lack of description about how the "traditional" RNAseq libraries were prepared.

**Thank you for pointing this out. In this methods paper, we have used the term “traditional RNAseq” to mean “only rRNA depleted, while “Capture” refers to enrichment steps with custom probes. We have inserted text on page 7, the end of Step 4 to clarify this as follows:**

**“Note: This library is considered a RNAseq library. Subsequent steps lead to a Capture library.”**

6. In the Data Analysis section, part 6.2 RNA-seq QC, how was the BAM file generated before alignment? I guess RNA-seq QC should be done after alignment.

**For analysis methods, please revised text on page 10.**

7. In the Data Analysis section, part 6.4 Variant Calling, if use STAR as the mapper for variants calling, it should be cited.  
**For analysis methods, please revised text on page 10, STAR is now referenced.**

8. Abbreviations should be consistent throughout the paper, such as RNA-seq vs RNAseq.

**Thank you for this comment, we have revised on page 10, item 6.2, to be consistent with “RNAseq.”**

*Additional Comments to Authors:*  
N/A  
  
  
**Reviewer #3:**   
*Manuscript Summary:*   
This manuscript describes a pipeline for the realization and analysis of targeted RNA-seq experiments. It describes both the wet lab and dry lab parts.  
  
*Major Concerns:*  
The described pipeline applies only to human RNA-seq experiments. For other organisms the reference genome should be changed. This could be stated either in the manuscript or in the title.  
**That is correct, this RNAseq assay could be adapted to other organisms, and the reference transcriptome should be accordingly utilized instead of human reference.**

As a statistician I mainly focused on the dry lab part. The description of the pipeline lacks important information such as :  
**Thank you for these comments.**

- the type of quality control metrics that should be computed and how to interpret them. In particular, are there cases for which the analysis should be stopped for quality reasons ?  
- which version of the softwares included in the pipeline should be used ? With which parameters ? This is important since with GATK (which is used for SNP detection) only recent versions apply to RNA-seq data analysis. So the authors need to specify the version that is suited for this pipeline.   
**For analysis methods, please revised text on page 10.**

**Each software manual includes suggested quality metrics that could be followed standardly. We have not detailed them in this methods paper. This is outside of the scope of demonstrating targeted RNAseq.**

**Editor: For analysis methods, we have provided the same level of detail as seen in JOVE article Won et al., Detecting Somatic Genetic Alterations in Tumor Specimens by Exon Capture and Massively Parallel Sequencing. More than this would amount to 10+ pages of text.**

*Minor Concerns:*  
None  
  
*Additional Comments to Authors:*  
N/A