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| Date: | Jan 19, 2016 |
| To: | "John B. A. Okello" jbao@queensu.ca,jbaokello@gmail.com |
| From: | "Nam Nguyen" nam.nguyen@jove.com |
| Subject: | Revisions required for your JoVE submission JoVE54299R1 |
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| http://www.editorialmanager.com/jove/img/paperclip_icon.gif**Attachment(s):** | [54299\_R1\_113015.docx](http://www.editorialmanager.com/jove/download.aspx?guid=%7Ba32e49f7-30d3-4412-8b8b-c5895b56a7f6%7D&scheme=13&id=6173) |
| Dear Dr. Okello,  Your manuscript JoVE54299R1 "Extraction of both RNA and DNA from formalin-fixed paraffin embedded tissue cores" 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 Feb 02, 2016. 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](http://www.editorialmanager.com/jove) and log in as an author. You will find your submission under the heading 'Submission Needing Revision'.  Sincerely,  Nam Nguyen, Ph.D. Science Editor [JoVE](http://www.jove.com) 1 Alewife Center, Suite 200, Cambridge, MA 02140 tel: 617-674-1888 [https://www.jove.com/files/img/signature/jove_signature_twitter.png](https://twitter.com/JoVEJournal)[https://www.jove.com/files/img/signature/jove_signature_facebook.png](https://www.facebook.com/JOVEjournal)[https://www.jove.com/files/img/signature/jove_signature_linkedin.png](http://www.linkedin.com/company/jove) [https://www.jove.com/files/img/signature/jove_signature_jove.png](http://www.jove.com/) \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_  Editorial comments:  The manuscript has been modified by the Science Editor to comply with the JoVE formatting standard. Please maintain the current formatting throughout the manuscript. The updated manuscript (54299\_R1\_113015.docx) is located in your Editorial Manager account. In the revised PDF submission, there is a hyperlink for downloading the .docx file. Please download the .docx file and use this updated version for any future revisions.  Changes made by the Science Editor:  1. There have been edits made to the manuscript.  Changes to be made by the Author(s):  1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.   * The manuscript has been edited and grammatical errors corrected as seen in track changes.   2. Please verify the in step protocol references.   * The references have been verified and a few errors corrected as seen in track changes.   3. Formatting: Short Abstract – Unless the validation steps are detailed in the protocol, the second sentence should be removed.   * Since the validation steps are not detailed in the protocol, the second sentence has been removed as suggested.   4. Grammar: -Line 156 – “protocol” should be plural   * Corrected as suggested.   -1.1 – Please correct grammar in the first sentence, which is a run-on.   * A period introduced in the sentence to avoid the run on error; splitting the sentence into two as shown in track changes.   -1.3 – The last sentence is incomplete.   * A period has been introduced at the end of this sentence, and the hanging word “and” deleted.   -6.6 – Last sentence should end with a period.   * Done.   5. Visualization: Step 1.5 must be highlighted. It is unclear why this entire section except for the main action is highlighted.   * Highlighted as suggested.   6. Unnecessary branding should be removed: The last column of Table 1 can be deleted due to branding.   * The last column in Table 1 has been deleted.   7. Results: All figure legends should have a title and a brief description. Figure 1 lacks a description and Figures 2 & 3 lack titles.   * We have made changes to the figure legends, and each now has a title and a short description.   8 Discussion: Please discuss potential modifications and troubleshooting that can be performed with this protocol rather than modifications on the manufacturer’s protocol.   * We have revised the discussion to include potential adaptation of this protocol for LCM and cut-section samples as well as its implementation on automatic robotic extraction systems.   Reviewers' comments:  **Reviewer #1:** *Manuscript Summary:* This article focuses on the extraction of RNA and RNA from FFPE tissue cores. The protocol is very detailed yet easy to follow. The Authors also present an excellent analysis of analyte yields based on a variety of factors. This article will be an excellent reference for those working with these analytes as FFPE banks represent rich sources of patient tissue. The accompanying video will be of great benefit for guiding investigators new to these techniques.   * We are gratified by the reviewer’s endorsement of this work, and will work with the JoVE Editorial team to ensure the key steps in the protocol are captured in the accompanying video.   **Reviewer #2:** *Manuscript Summary:* This is a well described method for the extraction of RNA and DNA simultaneously from FFPE tissue cores from samples. The use of modified commercially available kits/protocols should put this methodology within reach of any laboratory with a reasonable amount of expertise in standard molecular biology techniques. It should be noted that this work still requires the expertise of an experienced pathologist as selection of appropriate regions for nucleic acid purification remains the most important step in this process. The figures are clear and adequately show the utility/yield of this procedure for samples dating back up to 15 years. The detailed reagent list is helpful. While not specifically tested for this paper, the authors describe nucleic acid yields that should be useful for next-generation sequencing experiments and other downstream applications.   * We thank reviewer #2 for the positive evaluation of our manuscript. We look forward to utilizing this protocol in next generation sequencing studies.   *Minor Concerns:* -Utility is somewhat limited using brain tissue, likely due to the high lipid concentration of this tissue, but this limitation is clearly presented in the text and figures.   * We concur with this observation.   -Margins of table one are cut off in my downloaded version of this text and should be verified.   * We will work with the editors to ensure that the final document is properly formatted. We note that complete images could have been viewed by clicking the appropriate links in the pdf version of the manuscript.   -Error bars should be added to figures 2 and 4c. They are discussed in the figure legends, but not visible on the graphs.   * Unfortunately, error bars would not be applicable here since the figures in question are showing individual sample/data points.   **Reviewer #3:** *Manuscript Summary:* An excellent and well written manuscript that explains in detail the rationale for using the extraction method as well as all the steps that are part of the process.   * We thank reviewer # 3 for this positive feedback.   **Reviewer #4:** *Manuscript Summary:* The manuscript described the use and optimization of a commercially available kit for simultaneous extraction for RNA and DNA from formalin-fixed paraffin-embedded (FFPE) tissues. The authors describe three modifications, including homogeneization in ethanol, use of an "improved" protein K, and digest time extension from 2h to 24h. The protocol is clearly described step by step and the authors use clinical specimens from gene expression and methylation analyses.  *Major Concerns:*  There are a couple major concerns with the manuscript: 1- In the abstract and later in the introduction statements relating to the lack of "validated methods for simultaneous extraction of RNA and DNA.." are inaccurate. The method developed by Kotorashvili et al. 2012 describes an improved approach to extract RNA and DNA simultaneously from tissue sections, and thus is most likely applicable to the extraction of tissue cores. This method stems from the protocol developed by Loudig O et al. 2007 initially developed with tissue cores that underwent homogeneization.   * We thank the reviewer for pointing out these additional methods paper and have improved our manuscript by citing them and their contributions in the text and in Table 1. We point out that neither of these published protocols addresses the main purpose of the method reported in this manuscript: To simultaneously extract both RNA and DNA from FFPE tissue cores.   2- The use of tissue cores represent a major issue for collection of heterogeneous cells as cells below the surface of the tumor or lesions may vary and allow introduction of normal surrounding tissue. The use of LCM is indeed time consuming and expensive, but the use of tissue core, at a time when tumor cell heterogeneity is being explored represents a global and potentially error prone approach. Comparisons between microdissected cells from randomly selected tumors and a tumor cores obtained from these tumors should be performed to determine the gene expression and genomic DNA methylation profile differences.   * We thank the reviewer for this observation and have addressed it in a new section in the revised discussion starting on line 495. In essence, this is a valid concern, but one that has been widely addressed in the tissue microarray literature. In the revised manuscript, we provide a reference to a pertinent review on the topic, along with an alert to the reader to consider this issue.   *Minor Concerns:*  There are actual concerns with the development of the method:  1- The use of a motorized homogenizer will allow for tissue particles to remain trapped in the instrument. A comparison between different homogenizations approaches and non-homogenization would provide a better sense on the improvement.   * We thank the reviewer for bringing up this concern. We submit that homogenization is a common practice in molecular biology, and that we not only include multiple wash steps and inspection steps, but also repeatedly alert the user to pay careful attention to avoid this pitfall.   2- The use of ethanol during homogenization may not be adaptable to tissue sections or selected tissue lesions from sections or LCM, for RNA purification.   * We agree with the reviewer on this issue and go back to the first “Major” point the reviewer brought up. This protocol is not meant to inform nucleic acid purification from LCM or sectioned samples.   3- The purification of RNA from the ethanol fraction recovered after homogeneization is somewhat puzzling as FFPE RNA has been shown to be cross-linked to proteins, hence the use of proteinase K for improved recovery. Once again an extraction comparison with and without proteinase K, followed by RTqPCR for a few genes, would allow to determine the amount of information lost or tissue expression specificity retention, as well as the amount of RNA lost. It is known that miRNAs will not cross-link in FFPE tissues and will wash off especially during FISH experiments, and thus will provide most of the RNA fraction recovered during homogeneization. Messenger RNAs however may remained trapped in the tissue and thus may not be extracted efficiently in this way. A Bioanalyzer profile observing RNA purified in both ways would somehow provide details on the selectivity of this ethanol based purification.   * We thank the reviewer for raising this issue. These are valid points about RNA quality from FFPET, but we submit that the “proof is in the pudding.” Indeed, in other work, we and others have shown that RNA from FFPET is highly fragmented, and cannot be reliably analyzed by Bioanalyzer. We therefore used quantification by a sensitive and specific fluorometric assay, as well as endpoint analysis (RT-PCR and NanoString) to validate the suitability of the RNA. In the revised manuscript we clarify this issue (line 380) and provide a relevant reference. .   4- The use of a high potency proteinase K that displays such difference with the commercially available enzyme is striking, but no information on its origin, purification (grade), concentration is provided, which leaves room for questions. This enzyme alone may account for most of the improvement alone. More detailed comparative measures between the pK provided with the All Prep kit and the high potency pK should be given.   * We agree that the information requested is important. Due to editorial instructions from the Journal, we have excluded this information from the main text, but include only what is allowed in the reagent list.   5-The difference between 2h and 24h of pK treatment does not appear to be significant, and no repeated measures can provide such consensus. Repeats should be provided.   * We thank the reviewer for pointing out this issue and have revised the text to downplay any differences between the 2 hour and 24 hour time points (line 374).   6- The methods used for quantification of RNA and DNA are not provided. It is noteworthy that amounts determined with nanodrop or other types of spectrophotometers are somehwhat inaccurate, especially working with FFPE DNA. The Qubit apparatus is a better and more accurate instrument.   * Nucleic acids were quantified flourometrically using the Qubit system. As instructed by the Journal, brand names are included in the reagents section. In the revised manuscript, we alert the reader to look in this section for details.   *Additional Comments to Authors:*  Considering that this study highlights the improvement of an already existing commercial kit, side by side comparisons with the initial protocol should be performed to provide users with a better sense of the improvements.   * We thank the reader for this suggestion and submit that our presentation would be more streamlined and useful for a user/reader than one that tried to compare tissue sections to cores. | |