…08.2016

Jaydev Upponi, PhD

Science Editor, *JoVE*

Dear Dr. Upponi,

**Re: JoVE55049R1**

**Cell-free DNA integrity analysis in urine samples** by Valentina Casadio et al.

We are submitting the above manuscript, fully revised in accordance with the reviewers’ suggestions, for further evaluation. As requested, we enclose a point-by-point reply to all the comments made by the reviewers and have marked all the changes made to the text in bold.

We look forward to hearing from you.

Yours sincerely,

**Valentina Casadio, PhD**

**Corresponding Author**

**Editorial comments:**

•Formatting:  
-Reference citations – Some superscript numbers are bold and in italics – please correct this.

**Reply: We have corrected reference citations throughout the text.**

-Ethics statement should not be formatted as a note, just a line without a bullet number. Please also use 12 pt font size.

**Reply: We have corrected the ethics statement (line 76).**

-1.4 – “, better, at 4°C” – if there is a preferred option, that should come first, and the second should be an alternative. If there’s a reason one is preferred over the other, that can appear in a note below the step.

**Reply: We have corrected the text as suggested (line 88).**

-Please include spaces between numbers and units.

**Reply: We have checked and included spaces between all numbers and units.**

-Please format table citations as “Table 1”, etc. and figure citations as “Fig. 1” etc.

**Reply: This has been done.**

-Please use the American standard of punctuation, with periods instead of commas as decimal points (see Table 2).

**Reply: This has been done.**   
-Line 265 – “Togneri et al” requires a superscript reference citation.

**Reply: This has been done, now line 274.**  
  
•Grammar:  
-Please copyedit the manuscript for numerous grammatical errors, some of which are indicated below. Such editing is required prior to acceptance and should be performed by a native English speaker.  
-Line 21 – “it proved to be most accurate” – please rephrase in a more objective tone  
-Line 32 – “Real Time PCRs on four sequences”  
-Line 37 – “this open the way”  
-Line 39 – “only few milliliters”  
-Line 54 – “it.The”  
-Line 66 – “One of the main goal of this method is that it is flexible”  
-Section 2 heading – “and cell line”  
-2.2, 2.7 – Please correct the run-on sentence.  
-2.9 – “all of the sample mixture are used”  
-3.1 – Should be “spectrophotometer”.  
-3.2 – “diluition”  
-Please do not use the personal pronoun “he”.  
-Line 213 – “The best cut-off” – this implies there are other cutoff values.  
-Line 223 – “reported divided”  
-Line 237 – “The first advantage is the possibility of studying urine as a source of biomarkers is non-invasive method and the protocol is simple” – please re-write and clarify

**Reply: The errors and queries have been addressed and rectified.**

•Additional detail is required:  
-1.3 – How are samples mixed?

**Reply: They are mixed by inverting the sample twice.** **We have added this information to the text (line 85).**  
-1.5 – What constitutes the upper part? Can an approximate volume be given? Is there a clear delineation between the upper and lower parts?

**Reply: The upper part to be transferred is constituted by a volume of 10 ml (two 5 ml vials obtained). There is no clear delineation between the upper and lower parts and we thus suggest leaving at least two ml above the cell pellet. This information has been added to the text (lines 90-91).**-Section 5 – Please provide a citation containing the details of the analysis.  
**Reply: We have added three citations (nos. 7-9) in which the analysis is described in detail.**•Branding: Please remove trademark symbols from the materials table.

**Reply: Trademark symbols have been removed.**

•Results:  
-Please provide a graph or table to support the claims in the last paragraph of the results section, in addition to a citation if this has been previously published.

**Reply: We have added a new table (Table3 ) to summarize the results obtained in the three different papers published by our group (the 3 references were already present in the original version of the paper (nos. 7-9).**

-Table 2 – If two replicates are being evaluated, please label the columns with replicate 1 and replicate 2.

**Reply: This has been done.**  
  
•Discussion: Please discuss the critical steps of the protocol as well as any troubleshooting/modifications that can be performed.

**Reply: In our opinion we have already discussed the critical steps of the protocol in the Discussion. Does the editor need some specific clarifications?**

•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.                 
                                        
•JoVE reference format requires that the DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.

**Reply: We were not able to find the DOIs for 2 references (nos. 2 and 12).**   
                                                   
•IMPORTANT: Please copy-edit the entire manuscript for any grammatical errors you may find. The text should be in American-English only. This editing should be performed by a native English speaker (or professional copyediting services) and is essential for clarity of the protocol and the manuscript. Please thoroughly review the language and grammar 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.

**Reply: The paper has been thoroughly reviewed for its English language content by a native English speaker.**

                   

**Reviewer #1:**  
*Major Concerns:*  
-At the beginning of the protocol, clarify the starting material and the main steps of the experiment. For example you could list: urine samples (aim: UCF DNA integrity analysis), cell line (standards), DNA extraction, DNA Quality and quantity control (spectrophotometer and Real-time PCR for a control gene-STOX1), Real-time PCR for specific oncogenes.

From this experimental plan I guess you need to perform two Real-time PCR (one for the control gene and one for oncogenes).

**Reply: As requested, we have added a brief summary of the protocol at the beginning of the section (page 2 lines 71-74).**

Why did you say three Real-time PCR (page 5, line 242)? Please, clarify.

**Reply: This was, in fact, an error on our part. We have substituted “three” with “two” (page 6, line 254).**

-In Representative results section: specify at the beginning that you performed an experiment starting from 5 samples (how many bladder or prostate cancer samples?)

**Reply: The experiment on 5 control samples was only made to test assay reproducibility. We have clarified this concept in the revised version of the manuscript (page 5, lines 208-210).**

Clarify Table 2: did you performed tho Real-time PCR wiyh the same 5 samples, so did you replicate the experiment?

**Reply: Yes, we replicated the experiment on the same 5 samples. We have emphasized this concept in the text (page 5, lines 208-210)**

In Table 2 what the values of the sencond and third columns are? Ct values obtained from two indipendent experiments?

**Reply: The values of the second and third columns are expressed as ng/µl and they were obtained in two independent experiments. We have added this information to Table 2.**

-In Discussion section: page 6 lines 259-262  
Specify the data separately for prostate and bladder cancer and cite the literature.

**Reply: In the sentences in question, we were only referring to our study on prostate cancer. The text has been modified to make this clear (page 6, lines 261-267).**

-page 6, Line 272: I don't think that at this point of the research you can infer to use UCF DNA alone

**Reply: We agree with the referee and have modified the sentence in question (page 6 line 280)**.  
  
Moreover:  
-Page 2, line 79: I suggest to refer to one sample, if not you can get confused. Mix samples...each sample or mix all the samples together (this hypothesis does not make sense, but to avoid misunderstanding, it is better if you speak about one sample. You can declare at the beginning of the protocol that you can perform the analysis starting from several samples

**Reply: We agree with the referee and have modified the text accordingly (page 2, line 85).**

-In Line 150 (Page 4) you say RT-PCR (Real time or Reverse trinscriptase? Please declare the abbreviation. Obviously it is a Real time PCR starting from DNA, performed to verify gene copy number, but if you are used to perform RT-Real time PCR for gene expression analysis, you can get confused for a while.

**Reply: We have substituted “RT” with “Real Time”.**

In regard of this point, it should be better explained the expression "regions known to be frequently amplified in solid tumors" (Abstract and page 2, lines 62-63): is it a DNA amplification? Please clarify or at least cite literature such as Schwab, "Oncogene amplification in solid tumors"; 1999, where there is a good definition: "The term gene amplification refers to the selective increase of the gene copy number and is better designated as DNA amplification. It should not be confused with elevated gene expression". In this way, since the beginning of the article, it would be clear that you refer to Real-time PCR from DNA, to verify gene copy numbers.

**Reply: We were, in fact, referring to DNA amplification. We have substituted the sentences in question with “regions known to have an increased DNA copy number” in order to avoid confusion with gene expression (page 2, lines63-64).**

-Page 4 , line 178 and Table of Materials:  
you say that the analysis can be performed using another PCR real-time instrument. Can you say the same for DNA extraction kit or Real-time PCR kit? Did you perform experiments with other kit and chose these ones?

**Reply: We only tested the DNA extraction kit and Real Time instrument mentioned in the Table of Materials . However, we imagine that other kits or instruments would achieve the same results.**

-Table 1: indicate Reference sequence for each gene (e.g NG\_007503.1 for HER2) on which you have designed the primers, the complete gene name and use the official gene symbol ([*e.g.*](http://e.eg/) ERBB2, not HER2)

**Reply: We have revised Table 1, adding the complete gene name and official gene symbol (first column) and the reference sequence (second column).**  
*Minor Concerns:*  
-Both in the abstract and in the text declare the abbreviation of urine cell-free DNA (UCF DNA) the first time you use this expression and than always use the abbreviation.

**Reply: This has been done.**

-Page 2, line 62 and 67: genomic regions

**Reply: This has been done.**-Page 5, lines 237-238-239 Check English

**Reply: This has been done.**  
-Check formatting of the bibliography

**Reply: This has been done.**  
-Check formatting of Table of Materials

**Reply: This has been done.**-Check double spaces in the text

**Reply: This has been done.**

**Reviewer #2:**  
*Major Concerns:*  
-The authors have previously described this procedure in three other research articles, as they correctly reported in the paper (PMID 23141783, PMID23509700, PMID26412928) focusing on prostate cancer samples and bladder (PMID23141783).  
-They enlarge the cohort to 314 urine samples, composed by prostate and bladder cancer patients, healthy and symptomatic individuals with benign disease of urogenital tract. Therefore, it is not clear how many samples are new and how many came from previous published results (cited before and in the references), probably a table (or supplementary table) could help the reader.

**Reply: We agree with the referee that this was not clear. All the results came from our previously published papers. We have added a new table (Table 3) in which we provide details of the studies.**

-STOX1 amplification or not, was suggested as control,the authors wrote "STOX1 is a gene that is not amplified or deleted in prostate and bladder cancer…". If they are sure they could use a reference, or I suggest to modify the sentence in "STOX1 is a gene that is rarely amplified or deleted in prostate and bladder cancer…" or "Amplification or deletion of STOX1 gene has not yet been reported in literature"

**Reply: We have substituted the sentence in question with “As there is little information available about the amplification or deletion of STOX1 in bladder and prostate cancer, this gene could be used as a control sequence for these tumor types” (page 5 lines 217-218).**

*Minor Concerns:*  
Typographical errors:  
-The abstract 13th line lack of a "T".

**Reply: This has been corrected.**

-Line 266 (page 6) please insert citation followed Togneri et al.

**Reply: This has been done (ref. no. 4).**