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To Dr. Jaydev Upponi Ph.D.

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Dear Dr. Upponi,

Thank you for your feedback and review of our manuscript “**Detection of low copy number integrated viral DNA formed by in vitro Hepatitis B Virus infection**” (JoVE58202R1).

We appreciate the time, effort, and comments given by the reviewers and have revised our manuscript to address each of their concerns. We have provided a point-by-point rebuttal below. Further, we have edited some sentences throughout the manuscript to improve clarity. Finally, we have resubmitted our Figures as high-quality .TIF files suitable for publication.

Thank you for considering this work for publication.

Yours sincerely,

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*Editorial comments:*

*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.*

*2. Please use American English.*

*4. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.*

*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.*

*For example: AmpliTaq Gold, Qiagen, Eppendorf, etc.*

*10. Please do not abbreviate journal titles.*

This manuscript has now undergone thorough proofreading and editing. Spelling has been altered to American English throughout the manuscript. In addition, the minor changes with regard to removal of commercial language and full journal titles have been implemented.

*Editor, 3. Unfortunately, there are a few sections of the manuscript that show overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please see lines: 42-44, 54-65, 90-95,*

*Reviewer 3, Minor Concern:*

*It is very similar to the paper recently publishes "Hepatitis B virus DNA integration occurs early in the viral life cycle in an in vitro infection model via NTCP-dependent uptake of enveloped virus particles.*

*Tu T, Budzinska MA, Vondran FWR, Shackel NA, Urban S" in J virol.*

As already mentioned in discussions with the editor prior to initiation of writing, this manuscript is an update to a previous protocol description (*Chapter 9 - Detection of Hepatocyte Clones Containing Integrated Hepatitis B Virus DNA Using Inverse Nested PCR*, Springer Nature, 2017) and the invitation for writing this manuscript was a result of our publication (*Hepatitis B virus DNA integration occurs early in the viral life cycle in an in vitro infection model via NTCP-dependent uptake of enveloped virus particles*., J Virol, 2018) allowing us to more thoroughly describe our protocol. Therefore, we must necessarily cover much of the same ground as these publications. In some instances, there are only so many wording rearrangements that can be implemented before clarity is sacrificed. Further, some of the terms highlighted represent those commonly used in the Hepatitis B Virus field (e.g. covalently-closed circular DNA) and cannot be rewritten without confusion. With these challenges in mind, we have now re-written these sections as much as possible with original wording.

*Editor, 6. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.*

We are unsure which specific steps the editor refers to in this comment. We have reviewed each step and feel we have now given ample detail for each step in the revised manuscript (on par with the example manuscript provided to us by the editors).

*Editor, 7. 1.1: What are the culture conditions?*

We have already provided multiple references for the detailed cell culture conditions to avoid any descriptions that are repeated from previous publications (as was criticised in point 3). Could the editor please outline what specific details are missing in this step?

*Editor, 8. Please specify what happens after centrifugation. Aspiration?*

We assume the editor refers to a missing step after Step 2.16. We have now added an extra step, which reads as follows: “2.17. Remove the supernatant by aspiration with a P200 pipette.”

*Editor, 9. Please expand the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.*

*We need more than one sentence per figure.*

*Reviewer #1: How about possibilities of false positive and/or false negative result by invPCR?*

*Reviewer #4: 3. The authors do not provide descriptions of positive, negative and contamination controls. This should be supplied.*

We had followed the format given in the example manuscript sent to us by the editor where only a single sentence was given per figure. We have now added three extra paragraphs in the Representative results (Lines 331-354 in the updated manuscript) regarding expected results in the controls, as well as additional information to analyse sequences for false positive results (Step 5.2., Lines 301-318). If this is not sufficient, it would be helpful to know exactly the structure of the representative results required from us by the editor with another more accurate example.

*Reviewer #1: In line 89, in Introduction; cassette ligation PCR is one of the useful method for HBV integration. The below paper should be referred.*

*Tamori A et al., Alteration of gene expression in human hepatocellular carcinoma with integrated hepatitis B virus DNA.*

*Clin Cancer Res. 2005; 11: 5821-5826.*

*Reviewer #4, 6. Regarding introduction (line 76), this reviewer noticed that the authors forgotten to mention that a very similar model applying patient originating HBV and HepaRG cells has been recently established and the applicability of the invPCR approach to detect very early integrations of HBV and woodchuck virus to host genomes have been documented (Oncogenesis 2017). The authors should add this reference.*

We apologise for overlooking these studies and have now added these references in Lines 85-91.

*Reviewer #2: My only (and very minor) critique is that the "untreated samples" comment in line 314 is a bit vague.*

This comment has now been addressed and we have greatly expanded this section with paragraphs describing the expected results from positive samples (lines 331-343):

“**Expected results from positive controls**

Huh7-NTCP cells infected with heparin-purified HBV stocks as described above (**Figure 1**) can serve as a positive control. In this instance, single PCR products should be achieved by the second or third 1:3 dilution of each sample (corresponding to ~104 cell equivalents per dilution, **Figure 2**). At these dilutions, ~50% of products will represent true virus-cell DNA junctions (**Figure 3A**), while the other half represent amplification of HBV DNA intermediates (**Figure 3B**).

In previous studies13, we have found few instances of repeated virus-cell junctions, suggesting no cellular genomic sites of preferential HBV DNA integration. We also find that >80% of virus-cell junctions occur between nucleotides 1732 and 1832 (according to the nucleotide numbering of HBV genotype D, GenBank Accession #U95551.1), the latter representing the right-hand terminus of the HBV dslDNA form. Sequences of true virus-cell junctions found by our method in *in vitro* experiments are publically available on Genbank (Accession numbers MH057851 to MH058006).”

*Reviewer #3: On Figure 3 , in addition to the schematic figures, readers may appreciate to get informations on the nature of the genes where insertions are located, same for virus-virus jonctions some more informations may have been provided on what they are.*

*Reviewer #4, 2. The statement should be made regarding the rate of nonspecific invPCR amplifcation, i.e., frequency of bands which do not carry virus-host junctions after completion of analysis.*

Both of these points have been made in our previous publication and we have had internal discussions on how much to include in this protocol description (particularly as we have sought to minimise repetition with previously-published works, as we have been criticised for by the editor, point 3). We have now added additional sentences to the discussion: “We have found that ~90% of amplified sequences represent HBV DNA rearrangements (and not integration events) in terminally-differentiated cells, compared to 70-50% in hepatoma cell lines. These products are generally HBV DNA genomes containing large deletions in the surface and core open reading frames, or represent HBV quasispecies with an additional *Nco*I site prior to the *Sph*I site. The amplification of these HBV species (including a schematic diagram) have been extensively described in detail previously.”

*Reviewer #4, 1. The manuscript does not describe methodology for identification of virus-host junctions at the single bp resolution. Which computational approaches are/have been applied and what are criteria have to be considered during analysis? This additional information should be provided.*

*Reviewer #4:4. How is a number of positive hits of a particular virus-host junction calculated using the approach described? How can be it learned if a particular junction is a preferable site of HBV integration? Please explain.*

A separate section (Section 5, Lines 294-323 in the revised manuscript) has now been added and specifically outlines how virus-cell junctions are determined by BLAST analysis and analysed (with respect to filtration criteria). This section also include a paragraph on the determination of unique integration events and analysis for repeated integration into preferential cellular regions: “Define unique integration events as those with the exact HBV and cellular sequences at the virus-cell junction. Repetition of unique integration events can be due to clonal expansion of cells containing those integrations, or cross-contamination during the PCR (which can be tested using negative-control reactions, further detailed below in the representative results section). Repeated integrations into specific cellular sequences would be expected to display different HBV terminal sites for each integration event as using non-homologous end-joining pathways are used.”

*Reviewer #4, 5. The invPCR approach presented is limited to finding junctions fused with relatively small fragment of HBV DNA. Please explain to the audience reasons why this region was originally selected for priming. In addition, please comment whether it is feasible to design invPCR conditions amplifying host genome junctions with other regions of HBV genome.*

We have now outlined the reasons behind this design (and its weaknesses) in the discussion of the revised manuscript (Lines 426-430): “Moreover, our inversion protocol is only suited to detect integrations occurring between the DR2 and DR1 region of the HBV genome as the majority of HBV integrations occur within this region. NGS analysis of HBV patient tissues has showed that a large minority (up to ~50%) may also occur outside of this region48. New invPCR designs are theoretically possible to detect these other integration sites, though have not (to our knowledge) been carried out yet.”

*Reviewer #4, Minor Concerns: Fig 2 (lower panel) gives an impression that the bands were not completely cut out from gels. Is it artifact or the bands do not need to be removed from gel completely?*

In Figure 2, the bands are in fact excised completely. We believe that the lightening around the area of the excision hole that the reviewer refers to is simply an unavoidable artefact caused by UV light reflection against the cut surface and does not represent residual DNA in the gel.