18/01/16

Dear Dr. Upponi

Thanks for the feedback.

All comments have now been addressed in a line-by-line response (text in blue) and manuscript edits are marked as tracked changes.

***Editorial comments:***

*•****NOTE: Please download this version of the Microsoft word document (File name: 54239\_R1\_112315) 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.*

The format was maintained.

*•****Audio issues***

*• The audio in this version sounds noticeably more compressed than the previous submission. If this was added intentionally, the compression should be removed. If it was done inadvertently due to an export setting, that should be corrected when the next version is exported.*

The audio equalizer was altered to improve export of audio. We have made alternative sound versions that we will be happy to supply in case you prefer other settings.

**•Commercialization**

• 4:51- The shot of the kit insert should be cropped so that the brand name is not visible.

The shot of the isolation protocol was deleted.

• 8:25 - The soundbite naming Illumina HiC and the link to the illumina site should be removed.

The bite was deleted.

•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.

All figures are original and have not been published elsewhere.

•\* JoVE reference format requires that 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.

Missing DOIs are now included when available.

•***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.***

A native English speaker has reviewed and edited the manuscript.

**Reviewers' comments:**

**Reviewer #1:***Manuscript Summary:*Recommend for publication after minor revisions.

*Major Concerns:*

*N/A*

*Minor Concerns:*

*In the video, the introduction about recombination is not really necessary, as well as showing the growth of cultures.*

To maintain focus on the method we moved the model presentation to part 6 (representative results), where it serves as explanation for illegitimate homologous recombination and how some circular DNAs may form. We also made part 1 shorter by excluding video clips of initial growth and absorbance measurement.

*It is not clear which type of microscope was used to visualize PI-stained DNA and at which settings/magnification.*

The equipment for fluorescence microscopy is mentioned in Table S1 (Nikon Optronics Magnafire). The method for PI-stain was added to Table S1:

“Images of propidium iodine stained DNA were captured by fluorescence microscopy at 100x magnification (100x/1.30 oil, Nikon) in the RFP channel (red excitation fluorescence filter, 663-738 nm) using identical exposition time (5 seconds).”

As well, to step 3.4.3.3) was added: “at 100x magnification” and to line 358 was added: “See Table S1for extra details”.

*Additional Comments to Authors:*

*N/A*

**Reviewer #2:**

*Manuscript Summary:*

The manuscript by Bojsen et al describes in detail a method to purify extrachromosomal circular DNA from yeast. The method uses glass bead dependent perforation of the S. cerevisiae cell wall, kit based DNA purification, limited digestion of chromosomal DNA, extensive digestion of linear DNA and rolling-circle based amplification of DNA, prior to sequencing. Although the method is designed for yeast it could be adopted to all eukaryotic cells. The method as such has been published, but the video supported detailed description will proof very useful to the scientific community, especially since the protocol describes the reasoning of the method and explains experimental pitfalls. The video is in general of high quality, but the sound quality is poor and should be improved. The video has a nice balance of content to detail and is nicely done. The illustrations are of good quality and help with the explanation. That said, I am not sure why the restriction enzyme digest was not listed in the overview? In summary, I fully support the publication of the video-manuscript.

We thank you for your comments. The sound quality was compressed at export and is improved in the present version of the manuscript. The restriction enzyme digest was not listed in the movie intentionally as this is an optional step in the protocol. We have however listed the restriction enzyme treatment in the written manuscript.

*Major Concerns:*

I do not have any major concerns with this publication.

*Minor Concerns:*

-Line113: It would be useful to mention that the 86 kilobase DNA is of mitochondrial origin.   
We now mention 86 kilobase DNA is of mitochondrial origin in the introduction. It is also stated in the discussion as well as in reference 19.

-Why did the authors choose to limit their circular DNA's to a size of >1kb?

The >1kb cut-off was basically a choice to focus on large circular DNAs that could include full genes. We have now explained this in the introduction: “A size cut-off was chosen to focus on eccDNA that are large enough to carry whole genes”

-Line 118: It is stated that the method is sensitive and can detect 1 circle in thousands of cells. Is it possible to estimate how many circle with any given sequence exist per cell?

Currently, the best estimation is obtained by adding control plasmids to a known number of cells. To estimate how many circles exist per cell is very difficult because the phi29 amplification is biased toward small and abundant circular DNA’s. Also, we do not expect an even distribution of eccDNAs in cells, why we are reluctant to give an estimate.

-Line 129: yeast peptone (not pentose) dextrose. This has now been corrected.

-Line 130: the movie states 0.012 OD600. This clip was removed and we added “approximately” to line 134.

-Line 139: To spin down yeast at 420 g appears very low - needs checking. Thanks for noticing. The cells were pelleted at 2000 min-1 in a swing bucket Sorvall Heraeus Multifuge. radius 18 cm. Line 143+146 were changed to: “800 x g.”

-Line 150: Is it useful to indicate the specific volume of plasmid stock mixture? What is the definition of sample?

A stock mix for 20 samples was made (155 µl volume) but we don’t find this information important. The note on line 154-157 was revised: “NOTE: In the current dataset, a 7.7 µl plasmid mixture was applied for each sample containing 1010 cells. The plasmid stock mixture consisted of three plasmids in different concentrations; pBR322 at 38 ng/sample, pUC19 at 0.5 ng/sample, and pUG72 at 0.01 ng/sample.”

-Line 181: Short term storage of DNA in water is OK, but acid hydrolysis limits the long term stability. Would 10 mM Tris pH 8.0 be better? Water was used to avoid potential problems with downstream enzymatic steps. It might work equally well with 10 mM Tris pH 8.0 but we have not tested that. After part 2.6 in the manuscript was added: “NOTE: Only short term storage of DNA in water is recommended. Preferentially, proceed directly to step 3.”

-Line 186: Replace "Run" with Incubate. Corrected.

-Line 217: How much genomic DNA was used? Part 3.4.3.1) and 3.4.3.2) was revised to include template amount for control: “3.4.3.1) Use 2 μl exonuclease-treated sample as PCR template with *ACT1* primers 5'-TGGATTCTGGTATGTTCTAGC-3' and 5'-GAACGACGTGAGTAACACC-3'. As positive *ACT1* control, use 50-100 ng genomic *S. cerevisiae* DNA as template. PCR reaction conditions; 3 min at 95°C, followed by 35 cycles of 30 sec at 95°C, 30 sec at 56°C and 1 min at 72°C.

3.4.3.2) Run PCR reactions by gel electrophoresis on 1% agarose with 0.5 μg/ml ethidium bromide. Look for a 0.8 kilobase *ACT1* band.”

-Line 253: In the movie more info was given. Expand? We compared and did not find any crucial information left out from the text relative to the video.

-Line 362: Glass beads have been used to break the yeast cell wall, but they are also known to cause shearing of DNA. How was this balanced? The DNA is protected in the nuclei. In case of DNA shearing, we expect it will mainly target chromosomal DNA. The sheared DNA will facilitate digestion of linear DNA so we are not worried about using glass beads. In any case, we also suggest another disruption approach (line 166-167) by zymolyase, which should cause less DNA shearing than glass beads.

-Lines 362 to 365: It would be useful to compare the new method to the protocol from the Dutta lab and explain in which way the new method works different/better.

Dutta and her lab members tested their method on yeast and did found not circular microDNA from this organism. We speculate that the Dutta set-up might cause degradation of eccDNA or a methodological artifact, such as limited lysis of the yeast cells. Because the yeast protocol is not described in sufficient details, we cannot discuss differences between Duttas set-up and ours. A short sentence summarizing Duttas results on yeast has now been included in the introduction: “Dutta and coworkers also attempted purification of microDNAs from *Saccharomyces cerevisiae* but were unable to record microDNA from this yeast species16.“

-The inclusion of the tables into the PDF is problematic. Sorry. It was an automated PDF conversion of the Table. It will be solved for the final production.

-Video part 3: The figure is labelled DNase, but it is described as Exo. The label was changed in the video.

-Video part 4: Associate "Professor" is spelled with only 1 f. This error has now been corrected.

*Additional Comments to Authors:*

N/A