Rebuttal

Editorial comments:

General:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of 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: GlutaMAX, TrypLE, Qiagen, Qubit, ThermoFisher etc.

Protocol:

1. Everything in the protocol (except for the introductory ethics statement) should be in a numbered step (in the imperative tense and of no more than 4 sentences), header, or 'Note'. Please move the introductory paragraphs of the protocol to the Introduction, Results, or Discussion (as appropriate) or break into steps. Please avoid bullet points.

2. There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. If revisions cause the highlighted portion to be more than 2.75 pages, please highlight 2.75 pages or less of the Protocol (including headers and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

3. For each protocol step/substep, 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. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Specific Protocol steps:

1. Please combine 1A/1B and 2A/2B into single sections; the numbering is currently somewhat confusing.

We have adapted the protocol in a way a single sequence of steps can be followed. Depending on input the type and condition of input material different steps can be skipped. In addition, we have added a flowchart indicating the order of the experimental steps for which tissue.

2. 10.3, 10.4: How exactly do you run these scripts (i.e., the exact command)?

We thank the editors for pointing this part out. We added the execution command in step 10.4 which uses the the .ini file edited at step 10.3.

3. 10.5: How exactly is this done?
We have more thoroughly explained the procedure and have given a more detailed description of the commands used.

Representative Results
1. Representative Results: Please include at least one paragraph of text to explain 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 (except those referenced elsewhere). Data from both successful and sub-optimal experiments can be included.

Figures:
1. Please remove the embedded Figures from the manuscript.
We have removed the embedded figures from the manuscript.

2. Please remove the embedded table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.
3. Figure 4: There should be a closing parenthesis after VAF.

We have corrected this typographical error.

References:
1. Please do not abbreviate journal titles.
We have changed our reference style and do no longer abbreviate the journal titles.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

We have double-checked the table of materials so it contains all used material and equipment.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The manuscript by Rosendahl Huber et al provides a well-written overview of a method that is likely to be of significant use to the research community. Overall the paper provides a clear protocol of the approach, which should be relatively

easily transferable to other labs. I have raised several issues that I think would be useful to address:

Minor Concerns:

1. The methods describe the procedure for human cells. Is there any reason to believe that the method for murine cells would be different? If not, please state this. If yes, it would be useful to get some insight into what additional challenges might be confronted (personally I believe the method should also work for murine cells, if the sorting strategy and the culture conditions are amended).

We thank the reviewer for pointing this out and agree that this method is not uniquely applicable to human material. Currently, we have not tested our approach on murine HSCs; however, in the past, our group has performed a similar assay on murine intestinal stem cells, see Extended data figure 8 (Blokzijl et al., 2016).We agree with the reviewer that the wet-lab protocol required amending the sorting strategy and culture conditions. Adaptation of scripts is required to determine mutations as well. We adapted the discussion to state the possibility of appending the protocol to mouse material.

2. Line 126: I would rewrite "When the sample IS almost thawed, wipe the vial with 70% ethanol and transfer ITS CONTENT to A 50 ml conical tube".

We have corrected the typographical errors in the text.

3. Line 143: may be it is useful to specify what a FACS buffer constitutes of?

We thank the reviewer for pointing out this ordering mistake and will bring the explanation (present at line 174) to line 143.

4. Line 148: It is little odd to only start culturing MSCs after step 1.9, where hematopoietic cells are essentially ready to be sorted. Obviously, MSC cultures should have started much earlier so that these cells are ready to go after step 1.10. I.e. some textual reshuffling is needed.

We agree with the reviewer the sequence of steps in the protocol is not optimal. We have revised the ordering of the manuscript. As some steps have to be performed simultaneously, we have added a flow-chart for a more clear representation of the laboratory procedure.

5. Line 160: How do you ensure that all hematopoietic cells are washed off? If they are not specifically depleted, these cultures will still contain a very significant number of monocytes/macrophages that are pretty sticky and will not be washed off. This may not be important for this specific application, but good to mention whether or not it is essential that ALL hematopoietic cells be washed off.

We thank the reviewer for pointing this out. During culture, we indeed observe some colonies will differentiate and stick to the bottom of the well. Due to our washing technique we usually all, or almost all cells are taken up, due to fiercely pipetting and scraping the bottom of the well. We have not mentioned the scraping of the well, which is addressed in the revision.

6. How many HSPCs can be stained with the antibody mix shown in Table 3.2? The text reads "spin down AT LEAST 10e7 cells, but that suggest that there is no maximum number, which is clearly not the case.

We thank the reviewer for pointing out this inconsistency. We have successfully sorted up to $2*10^7$ cells with our technique, and it is now mentioned.

7. Line 241: CD45- should be CD45RA-. Also, is CD90 used in the sorting protocol? The figures suggest it is, but in the text I do not read this.

We thank the reviewer for pointing out this typographical error and will change CD45 in CD45RA. We agree with the reviewer we use CD90 and CD49f to distinguish multipotent progenitors from long-term HSCs. This is now added in the representative results section.

8. How do you confirm after sorting that indeed a single cell was deposited in each well? Is there no visual inspection shortly after sort? What are the consequences if 2 cells end up in one well?

We understand the concern of the reviewer. In our culture plates it is very hard to distinguish the individual sorted cells, and therefore we do not perform visual inspection after sorting. If multiple cells end up in one cell and both cells expand, the variant allele frequency (VAF) will not be 0.5 anymore for mutations present in all cells, but lower. This can be identified a peak-shift towards lower frequency in the VAF plots from the SNVFI (step 7.6 and 7.7).

9. Why would you want to harvest the first cultures after 4 weeks, and culture the plate for an additional week to see whether new colonies emerge? Why not rather just harvest all colonies after 5 weeks?

We thank the reviewer for addressing this procedure and we now explained the rationale behind this step in the protocol. The first colonies will emerge after 3-4 weeks and can survive for several days in the medium. However, we observed that fully grown colonies quickly deplete the medium and change its' pH, inducing cell death. To ensure optimal DNA recovery, wells are harvested when full during the 4^{th} and the 5^{th} week.

Reviewer #2:

Manuscript Summary:
The manuscript by Huber and colleagues presents a detailed "how to" for the

determination of mutational burden in human hematopoietic stem cells, which will facilitate the determination for how mutational burden changes with age and disease. There are a couple of minor concerns that are listed below, but generally the presentation is clear and the methods straightforward.

Major Concerns:
none

Minor Concerns:

1. Regarding the code, the authors should alter the code presentation such that the recommended settings no longer require the user to manually input them. We usually build a file that is referenced to do this, so that the user can just hit a button and run the program.

We thank the reviewer for pointing this out. We have provided or referenced to .ini files where both recommended settings and paths to input files and output folders are indicated. We have removed the recommended input settings from the protocol, as these can be found in the .ini files.

2. Regarding the method, it would be nice to know the cell viability and DNA yield that they get from their method of banking mononuclear cells.

In our revision, we have provided numbers on cell viability and recovery after thawing of mononuclear cells.

3. They should better explain the logic for using MSCs or T-cells as germline controls. While using MSCs make sense, since this is a different lineage, how is the use of T-cells better than using a pool of HSC? Perhaps clonal hematopoiesis (such as in the elderly) could lead to the inability to discriminate a somatic mutation from a germline one, but is clonal hematopoiesis less likely to be observed in T-cells?

We agree with the reviewers that T-cells are not an ideal sample due to their hematopoietic origin. Ideally to dissect a developmental lineage tree based on somatic mutations acquired during life, a bulk sample which does not share a developmental origin with the hematopoietic system is required, such as MSCs or a buccal swap sample. However, when only peripheral blood is available, T-cells can be considered a viable option, which is also being used in other studies focusing on studying the origin of leukemia within the blood lineage (Shlush et al, Nature 2017).

4. In step 1.9, they use a volume of isolation rather than cell counts. The isolation is just for a flow control, but they are giving specifics on the antibody concentrations, and cell count differences will alter fluorescence intensities.

Therefore, it might be helpful to just specify the number of cells to use in the flow controls. Ok, so this is being pretty picky!

We thank the reviewer for pointing out this step in the protocol. We specified the recommended cell number ($1*10^5$) required for the flow cytometry control sample.

5. For step 10.6: If we understand this correctly, they are suggesting that those locations which show a number of different mutations would be considered a bit low confidence and should just be thrown out. If this is the case, we really don't like the idea that this would be a judgement call without specific criteria, and secondly manual inspection using IGF seems like an unsatisfactory way of eliminating these mutations as it might take days or weeks per sample. Perhaps this is a misunderstanding on our part.

We thank the reviewer for pointing out the unclear passage in our manuscript. We agree with the reviewer we were not specific enough in our description. We have now rewritten this protocol step to be more specific in our validation criteria.
It is noteworthy that processing of sequenced reads to obtain genome alignments and then to call variants is a complicated process. A number of factors, including sequencing errors, actual variants diverging the read sequences from the references, could influence mapping quality. The variants are called from resulting mapping data assuming the mapping is done properly. This assumption holds true at the regions with unique sequences and in the genome, but not with the low-complexity regions. Even with the unique regions, the number of variants in the sequenced sample could limit the precision and accuracy. Although those regions we do not trust the variant calling from is obvious by eye as shown in the new Figure 7, we cannot distinguish such regions automatically with our current method. Adding to the technical difficulty, only a low number of mutations, typically less than 10, will be shared amongst clones, as most mutations are unique for each stem cell. Therefore, it does not take much time to evaluate all shared mutations. The manual check we perform here is also to validate our filtering and to ensure we only include high confidence mutation since a single mutation can determine the position of a cell in the lineage tree.
We agree with the reviewer this is a judgement call; however, we hope to convince the reviewer with the above explanation and the newly provided IGV examples that our manual check is reasonable and straightforward.

We have now written this protocol step as: “Mutations are considered false when not present, when the mutation is present in the germline or when present in poorly mapped regions”

6. Table 3.4 is jumbled in the pdf we received.

We thank the reviewer for pointing this out. We have supplemental table 3.4 in a separate excel file.

Reviewer #3:

The manuscript described a pipeline about analyzing de novo somatic mutations in HSPCs by clone sequencing. I hope the following questions would be clarified before the manuscript published.

1. At the Step 3.1, the author pointed out 1x107 cells required at least. What are these cells? Are they PBMCs?

We thank the reviewer for pointing this step out. We are now more specific and alter the sentence to: '$1x10^7$ mononuclear cells are required.

2. At the Step 3.11, the single HSPC was cultured in the incubator at 37°C for 3-4 weeks. Is it necessary to refill medium? If yes, how often? Does it need to transfer cells from 384-well plate to 96-well plate for clone expansion? Still at this step, the author found 5%-30% of sorted cells would clonally expand. Are the success rates different for fresh cells or frozen ones? Is there any difference between the rates of bone marrow, cord blood or peripheral blood?

While a 4-week culture period without changing medium seems long, the cells are perfectly able to proliferate for up to 4 weeks. We have observed enhanced growth when passaging colonies in 96-wells plates, and this might enhance the DNA load of a colony even further.

Regarding the question about success rates for clonal expansion: We have not specified for which samples we see a lower clonal outgrowth, as we see higher colony outgrowth in bone marrow, while we observe lower colony outgrowth in samples from peripheral blood. We clarified this further in the Representative results section.

3. At both step 4.6 and 4.7, the author mentioned ">30% confluency" or a small visible pellet" as the marker for cell collection. However, this description is not very specific. Is it possible to use the number of cells instead?

We agree with the reviewer that the size description for cell harvest and cell numbers are not highly specific. However, the primary objective of colony outgrowth is to obtain enough DNA to perform whole-genome sequencing and this increases with the number of cells. For cell counting cells have to be sacrificed, losing more cells in the process. When colonies have a confluency <30%, we typically observe too low DNA yields (<50ng) to perform whole genome sequencing.