Dear dr. Steindel,

Thank you for reviewing our manuscript entitled "Transcription start site mapping using Super-Low Input Carrier-CAGE" by Nevena Cvetesic, Elena Pahita and Boris Lenhard.

In this letter we address point-by-point all of the comments raised by the reviewers and all editorial comments. In the revised version of the manuscript with implemented responses to reviewer's comments, all newly added and modified parts are marked in red. We marked the parts for video production in yellow.

Editorial comments:
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 thoroughly proofread.

2. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.
Trademark and registered symbols have now been removed from the Table of Equipment and Materials.

3. Please sort the Materials Table alphabetically by the name of the material.
We have now removed subsections in the Table of Equipment and Materials and sorted everything alphabetically.

4. 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: RNAClean, Superscript, RNase ONE, AMPure XP, etc.
Please remove "AMPure" from Figure 1 as well.
We have now removed commercial language from the manuscript and Figure 1, and used the most generic terms where possible. We have kept the exact reagent/product and company name, along with the Catalogue numbers in the Table of Equipment and Materials.

5. There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings 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. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
We have highlighted in yellow the essential steps of the protocol for the video (up to 2.75 pages in length).

6. Please ensure that the highlighted steps form a cohesive narrative with a logical flow

from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.
We have ensured the aforementioned narrative for the highlighted steps.

7. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
We have revised the text to avoid the use of any personal pronouns.

8. Please do not abbreviate journal titles.
Full journal titles are now written in references.

Reviewers' comments:
Reviewer #1:

Cvetesic and collaborators propose an improved method for the Cap analysis of gene expression (CAGE) technique. CAGE is a highly sensitive method to detect transcription start sites (TSS) and can also be used for accurate quantification of gene expression. In the recent years, it has been extensively used to fine mapping active promoter and enhancer regions, thus providing critical information for the understanding of the regulation of gene expression. In the present study, the authors describe the SLIC-CAGE, a method allowing to perform CAGE experiments from very low amount of cells. The use and validation of this improved method has been published elsewhere (Genome Res. 28 (12), 1943-1956).

Comments:

1. To illustrate theirs results the authors should show some comparative analyses between data obtained with their method and published experiments performed with standard protocols as well as representative screen shots. If possible, sequencing of low complexity libraries could be shown for comparison.
We thank the reviewer for raising this point. We have now added a figure (Figure 5) that includes the mentioned visual assessment of library complexity – distribution of interquantile widths and include a low-complexity library for comparison. We also now include a comparison of SLIC-CAGE and nanoCAGE (alternative low/medium-input method based on template switching) performance based on CTSS identification (ROC curves) and use a nAnT-iCAGE as dataset to define true CTSSs (Figure 5B, C). We have added a comment at the end of the representative results paragraph pointing to the added Figure 5.

2. How the different size of the synthetic carrier are generated?
Each PCR template (ten in total) is produced by using a common forward, but a different reverse primer (Table 2), leading to a different length of the PCR template to enable size variability of synthetic RNA carriers. This has now been added to the paragraph representative results for clarity.

3. Figure 2: add the legend to the top of each panel.
We have now added the legend to the top of each panel.

4. Bioanalyser profiles shown in figures 3 and 4 should show the size distribution (bp) instead of Time (S).
We have changed the x-axis as suggested to show size distribution in bp.

5. The sequences of mentioned primers (5'linker, TCT-N6, etc) should be shown as supplementary table.
The sequences are now added in the Supplementary Table 1.

6. What is the "USER" enzyme?
USER enzyme is the commercial name for a mix of Uracil DNA glycosylase and DNA glycosylase-lyase Endonuclease VIII (produced by NEB). We have removed the commercial

names from the manuscript (commercial names are now used only in the Materials List). We have substituted USER name with uracil specific excision enzyme.

7. Step 8: how the number of cycles is determined should be specified.
This is specified in the Note under 18.3 step. For clarity we have now added that the number of PCR cycles is equal to the Ct value obtained with the adaptor primer mix.

8. Typos:
a. Line 51: "is added to the sample of interest"
b. Line 77: incomplete sentence: "TSS information is also proving important for profiling healthy and disease tissue »
c. Line 185: incomplete sentences?
We have corrected the mentioned typos and thoroughly checked the manuscript.

Reviewer #2:

In this paper Cvetesic et al., describe a rather long technic: SLIC-CAGE which maps the TSS used in cells/tissues. SLIC-CAGE allows to perform CAGE (CAP Analysis of Gene Expression) using very low amount of starting material (ngs of RNA) and its performance is demonstrated in a paper recently published in Genome Research.

The present paper is well written, easy to follow and well described; a sufficient amount of details are given for the readers to reproduce the protocols.

I only have minor comments:

1. The authors could try to limit the use of the reference of Murata et.al (11) to allow the readers to only refer to their new paper in the future.
We thank the reviewer for the comment and have limited the use of the reference in the revised manuscript. However, as the protocol is already very long, we have to reference some of the steps from Murata et al 2014, as the manuscript would exceed the limit of the journal.

2. It would be very valuable to have paragraph describing he critical points in the protocols to which further experimentalists should pay particular attention.
Critical points in the protocol were already discussed in the very beginning of the Discussion paragraph. We have now expanded this section and added a step critical for the quality of the library.

3. Could the authors explain the rationale of diluting primers in 0.1XTE why not water or TE (just a curiosity of the reviewer) which may also hit readers.
We only dilute the linker components in 0.1xTE (Supplementary Material, and as described in Takahashi et al 2012), all other primers are dissolved in water. We have now made this clear in the manuscript. In general, 1xTE is avoided due to high EDTA concentration that can inhibit enzymatic reactions by chelating Mg^{2+} ions, therefore we rather use 0.1xTE. As it is crucial that the 5' and 3' linkers stay annealed, NaCl addition is more relevant for

stabilization of the annealed linkers. We follow the protocol that was originally described for the tagging CAGE version in 2012 Takahashi manuscript. We have now made it clearer that this protocol for annealing and dilution is taken entirely from that publication.

4. It would be interesting to have a paragraph dedicated to processing of sequencing data.
We agree with the reviewer that it would be good to have a paragraph dedicated to processing of the sequencing data. However, due to length restrictions we cannot include a full paragraph as the manuscript is already at the page limit. We have now added a sentence on mapping and direct the reader that processing of the data and downstream analyses are well described in the CAGEr package vignette and publication.