Reviewer's report

Title: Small nucleolar RNA U91 is a new internal control for accurate microRNAs quantification in pancreatic cancer.

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Reviewer: Jinghuan Li

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Generally, this manuscript compares four controls used in RT-qPCR quantification of miRNA expression in FFPE samples of pancreatic adenocarcinoma. The authors applied spike control, NormFinder software and comparison among tested endogenous controls, conclude that U91 is the most stable endogenous control, and therefore recommend it as a new normalizer of miRNA expression in pancreatic adenocarcinoma. The work is important and interesting as it can provide a practical reference for researchers when choosing a stable endogenous control for miRNA studies in pancreatic cancer.

The writing language is acceptable. However, the conclusion could be different after the data being further analysed and proper interpreted.

Major comments:

1. The authors have not explained the differences between spike control and endogenous control. The experiment and conclusion are largely based on spike control RNA. Question: ‘If you believe the spike control is the standard, then why do you have to find another control?’ However, spike RNA is a technical control commonly added to the sample before RNA extraction to control technical errors during RNA extraction and RT-qPCR process. It neither reflects the quality of the original sample nor quantity of input RNA. Especially in FFPE samples, RNAs are partly degraded. Researchers often found difficult to determine an endogenous control to normalize variations among samples. This difficulty makes choosing an endogenous control important and valuable.

2. The authors have not analysed the data adequately. Figure 2 and 3 show the similar confusion. In Figure 2a, the CT difference is small because spike controls were added equally. While in Figure 2b, c and d, the CT differences are big which could be caused by poorer quality of the control sample then the tumour sample. In another word, higher degradation or lower input RNA of control sample could lead to higher CT value then tumour sample. This principle is also applied to Figure 3.

3. The authors have not fully understood the data shown in the last paragraph of Result section, line 247-257, NormFinder identified ‘the most unstable control was the artificial spike’. This is an evidence of artificial spike RNA not being a suitable gene expression control especially in the FFPE study because it does not represent sample quality. In another word, taking a foreign intact RNA as a control to study variably degraded RNA is meaningless. In my opinion,
NormFinder data should be properly discussed and is much more reliable than simply relying on the foreign spike RNA in this case. There are also other softwares available including GeNorm and BestKeeper.

4. RNA quality and quantity should be accessed.

Minor comments:

5. In line 232-233, this is confusing: ‘
##CT=(CTmiRNA-(CTcontrol±n)tumor-(CTmiRNA-CTcontrol)normal.’

6. Figure quality and presentation needs to be improved. For example, increase the size of the text and insert the labels to make them self-explained etc.

Conclusion:

Overall, the data of all three endogenous RNAs are differ from that of spike RNA indicating that the spike RNA is not suitable as gene expression control due to sample degradation variation. However, the author selecting a new control RNA largely based on spike control is fundamentally unacceptable.

Level of interest: An article of importance in its field

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:

I declare that I have no competing interests.