**Reviewers' comments:**  
**Reviewer #1:**  
Manuscript Summary:  
The manuscript reports a suitable strategy for selection of optimal SINEUP RNAs from a collection of sequences encompassing translation start sites, using, as a reporter, the EGFP protein. The manuscript is well written and some limitations and caveats are properly discussed.

**Response:**

We are thankful to Reviewer #1 for reviewing our manuscript and appreciate the positive feedback.  
  
  
**Reviewer #2:**  
Manuscript Summary:  
This is a very clear and nicely written manuscript reporting a method to enhance protein expression from mRNAs. As the authors rightly point out, this method is unique in that it exploit synthetic antisense RNAs known as SINEUPs to enhance protein expression at a posttranscriptional level rather than alter transcription or stability of the target mRNAs. SINEUPs are bipartite long non coding RNAs containing a 5' binding domain complementary to the target mRNA and a 3' effector domain comprised of an inverted SINE repeat that positively regulate mRNA translation. Several papers have been published showing the feasibility and potential of this technology. This method offers a complementary approach to commonly used antisense-RNAs methods designed to silence genes at transcriptional or post-transcriptional levels, and an effective alternative to transgenesis for the overexpression of genes. As outlined by the authors, the method has clear translational potential for example in promoting antibodies production or correcting haploinsufficiency in loss of function diseases.  
  
One of the critical parameters for the method to work accurately and with minimal off-target effects is the design of the binding domain of the SINEUP. This article describe an in vitro high-throughput method for the identification of the best sequence for SINEUP synthesis that allows to determine the most effective sequences for further in vivo testing.  
  
Major Concerns:  
No concerns  
  
  
Minor Concerns:  
No concerns

**Response:**We thank reviewer #2 for accepting our manuscript with positive comments. We completely agree with the comments about the critical parameters of the methods and off-target effects of the designed BDs of SINEUPs. As mentioned in the manuscript and highlighted by reviewer #2, we should carefully check off-target effects of optimum SINEUP BDs by comprehensive screening of other transcripts and proteins by RNA-seq and ribosome profiling methods, however discussion of these methods are beyond the scope of this manuscript.

**Reviewer #3:**  
  
Manuscript Summary:  
This article mainly focused on the method of using Micro-well Image Cytometer to screen functional SINEUP sequence for further usage. In all, the abstract and research logic is very confusing and some important data is missing. Besides, some important references which reported the similar method should be cited.

**Response:**

We thank the reviewer for reviewing our manuscript and appreciate the constructive criticism. We are sorry if our explanation led to any confusion and dissatisfaction.

Major Concerns:  
1. The abstract contains too many description about the advantages of SINEUP technology. However, only one sentence has correlation with the research in this article.

**Response:**

With all due respect, we believe that all the “advantages” of SINEUPs technologies stated in the abstract, such as, positive control of target-specific translation post-transcriptionally within the range of 1.5 to 3.0 fold and high-throughput detection are sufficiently discussed in this article with relevant results (**Figure 3 and 5**).

We assume that the reviewer is referring to the SINEUPs “applications” mentioned in the **lines 51-57** (revised version). We agree that we described several applications here for the readers’ interest, but did not go into detail for every one of them. However, as this is a methodological paper, we focused mainly on the basic SINEUPs technology and demonstrated its application with the example of SINEUP-GFP, the protocol stated here can be employed to achieve many of the SINEUPs applications with no or small modifications based on the goal. Moreover, as it is beyond the current objective to discuss various SINEUPs research results, we have cited two original research articles for natural SINEUPs (Carrieri *et al.*, Nature 2012 and Schein *et al.*, Sci. Rep. 2016) in **lines 75 and 82**, and also referred to detailed studies covering various applications in **lines 84, 93, 100, 102** (**Reference no. 12-15, 17-19, 21**).

We have also discussed SINEUPs limitations in **lines 403-406** as-

“A limitation of this high-throughput protocol is that it is not suitable to screen BDs of SINEUPs in in vivo mouse models because the protocol measures the GFP integrated intensity only. As SINEUPs are natural antisense lncRNAs that act post-transcriptionally, they cannot be applied when the target mRNA is missing in the cells or tissue samples.”

2. (Yao et al., Nucleic acids research, 2015, 43(9): e58-e58.) and (Long H et al., Biotechnology letters, 2017, 39(2): 179-188.) reported a similar technology named RNAe with same design rule and function. These related works should be cited in reference.

**Response:**

We are sorry for missing these citations. Following the reviewer’s comment, we have added these citations in **lines 344 and 376** (**Reference 31-** Yao *et al.*, Nucleic acids research, 2015), and in **line 400** (**Reference 33**- Long H *et al.*, Biotechnology letters, 2017).

3. The author performed the research to select different designs of SINEUP, but they didn't give neither experimental evidence nor speculation that different BDs will affect SINEUP efficiency. Besides, (Yao et al., Nucleic acids research, 2015, 43(9): e58-e58.) had already performed that alternating the BD sequence will lead to efficiency change of SINEUP, which seemed to be ignore by the author.

**Response:**

We believe that we stated BD-dependent efficiency changes of SINEUPs in **lines 280-282** (first submission version) as “we screened 17 BDs of SINEUP-GFP by Western blot analysis and found that the optimum BD overlaps the AUG-KOZAK sequence of GFP mRNA” and cited Takahashi *et al*., PLOSONE 2018 (**line 281** in first submission version) for further experimental details. We thank the reviewer for notifying us about Yao *et al.*, NAR 2015. We have now cited this study (**Reference 31**) in **line 344 (revised version**) in context of- “Another independent group also screened the BD using a different method31.”

We have also referred to this study in **line 376 (revised version)** with context of- “While we used a specific high-throughput micro-well cytometer, which enabled detection of GFP fluorescence across the entire well, other cytometers with a similar detection range can be used31.”

4. The result in Figure 5 is very confusing. The author didn't give any information of what sequence they use, but the enhancement ratio is different compared to the result in Figure 3. More details about the experiment should be added.

**Response:**

We are sorry for any confusing explanation. We have described here SINEUP-GFP as the optimum BD, which is one of the candidate SINEUP BDs in our screening from Takahashi *et al.*, PLOSONE 2018. We addressed the comment with details in **lines 290-295** (revised version) as “Having demonstrated that SINEUP-GFP increases GFP translation, we detected GFP integrated intensity by the image cytometer. **The optimum BD** **of** SINEUP-GFP induced a 1.4-fold increase in GFP protein expression. Although we observed compression of signals from 2.6-fold (Western-blot analysis, see Figure 3A) to 1.4-fold (Imaging analysis, see Figure 5), the difference might be due to the calibration of the imaging instrument software.”

In addition, we cited Takahashi *et al.*, PLOSONE 2018 in **Figure 3 and 5** legends (**Lines 321 and 332** in the revised version).

5. The author speculated fusing target protein with GFP can help to screen functional SINEUP, but more useful details should be discussed. Besides, the discussion of in vivo screening should be simplified.

**Response:**

We thank the reviewer for this comment. To address this, we have removed **lines 291-292** (first submission version) and added more details regarding GFP-fused target mRNA screening approach in **lines 384-389** (revised version) as-

“Furthermore, it can be utilized to expand the search for effective SINEUPs targeting several mRNAs, designing different SINEUP BDs around AUG-Kozak region (see Figure 1), co-transfecting full length target mRNAs (5ʹ UTR-CDS-3ʹ UTR) fused with GFP mRNA in cultured cells to find the optimum SINEUPs, and subsequently testing BD candidates against endogenous mRNA in cultured cells and *in vivo* model animals, from humans and mice to other animal and plant species.”

In agreement with the reviewer’s comment about the *in vivo* screening, we have removed the statements in **lines 309-315** (first submission version) for the sake of simplicity.