**Editorial comments:**  
Changes to be made by the Author(s) regarding the written manuscript:

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.

**Response:**

We understand editor’s concern about spelling or grammar errors. The revised version of this manuscript has been checked and proofread by native English speaker to meet the standards of JoVE.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

**Response:**

The figures reused in this manuscript are Figure 3 and 5 which are taken and modified from the PLOS ONE article authored by all authors of this manuscript (Takahashi *et al*., PLOSONE, 13(2): e018322915). PLOS applies the Creative Commons Attribution (CC BY) license to articles and anyone is allowed to reuse and distribute the articles completely or partially under proper citation. We upload copy of this open access license policy of PLOS in the Editorial Manager account. Please find uploaded “Explicit copyright permission of PLOS. docx” file. We have cited the source in the **Figure 3 and 5 legends** **(Lines 321 and 332 respectively)** as suggested by the editor.

3. Figure 2: Please define error bars and asterisk symbol in the figure legend.

**Response:**

We thank the editor for pointing out this. We have revised this figure legend (**Figure 3** in the revised version) and added definition for the error bars and asterisk symbol in **Lines 319-320**.

4. Figure 4: Please remove this figure which shows only a specific equipment and contains commercial information.

**Response:**

In agreement with this comment, we have removed “Figure 4: Example High Throughput Micro Well Image Cytometer”.

5. Figure 5A: Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate figure Legend.

**Response:**

Thank you for bringing our notice to this error! We have added scale bars to this figure and defined the scale in the figure legend **(Lines 328-329)**.

6. Please spell out each abbreviation the first time it is used.

**Response:**

We have thoroughly checked and described the abbreviations missed before **(for instance, in Lines 41-42, 45-46, 69, 103, 107-108, 112, and few more)**.

7. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

**Response:**

We have changed all the units to SI as per the editor’s suggestion.

8. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

**Response:**

Thank you for calling our attention to this detail. We have made necessary changes in the text following this comment.

9. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

**Response:**

We have changed the numbering in the Protocol to match the JoVE style.

10. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

**Response:**

We have made sure to remove all the personal pronouns in the revised protocol.

11. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. Please move the discussion about the protocol to the Discussion.

**Response:**

We thank the editor for pointing out these errors. We have made the recommended changes in the protocol ensuring use of imperative tense throughout the protocol. We have included safety instructions within the steps or as notes (for eg.- **Lines 222-224, 250-252**). We have moved the statement in the lines 129-131 in the previous version to the discussion (**Lines 341-343** in the revised version).

12. In the JoVE Protocol format, “Notes” should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.

**Response:**

We are sorry for mistakenly overlooking these guidelines. Following the editor’s suggestion, we have edited the Notes in lines 138-141 and 149-151 (previous version) and merged it with current **step 2.1** and **2.2** respectively (**Lines 136-140 and 143-146** in the revised version). We have modified the Note in lines 208-209 (previous version) and elaborated this part in **Step 5.2 to 5.8** (**Lines 225-236** in the revised version).

We have moved the following Notes to the Discussion:

Lines 142-145 (previous version) → **Lines 347-352** (revised version)

Lines 202-204 (previous version) → **Lines 358-361** (revised version)

Lines 217-219 (previous version) → **Lines 365-368** (revised version)

Lines 230-231 (previous version) → **Lines 376-379** (revised version)

13. Lines 123-131: It is unclear as to whether these design steps can be visualized. Please consider unhighlighting these.

**Response:**

We understand editor’s concern, but wish to keep these design steps for the video as these are very crucial for the success of this experiment. We added additional figure as Figure 2 that shows tissue and cell specific transcription starting site of Parkinson disease protein 7 (*PARK7*) mRNA in **Lines 127-128**. We explained how we design BD using the information of transcription starting site inspecific cell in the figure 2 legend **(Lines 303-314)**. We believe that integration of these steps in the video will be informative for the users..

14. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. 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.

**Response:**

We apologize for missing the details of some of the steps and thank the editor for drawing our attention to this. In the light of this comment, we have carefully checked the protocol and extensively revised the steps involved in protein concentration check (**Step 3.4- Lines 162-180**), SDS-PAGE (**Lines 193-195, 210-214**), RNA extraction and DNase treatment (**Step 5- Lines 216-236**), cDNA synthesis and qRT-PCR (**Step 6- Lines 238-264**), and imaging analysis (**Step 7- Lines 266-278**).

15. Lines 132-133, 207-213, 226-229: Please ensure that the protocol here can stand alone. As currently written, users must refer to elsewhere in order to complete this protocol.

**Response:**

Lines 132-133 referred to custom synthesis of user-designed SINEUP expression vectors. As synthetic SINEUP technology is part of the filed and granted patents [Functional nucleic acid molecule and use thereof; US9353370B2, EP2691522A4 and JP2017169573A], and Cell Guidance systems Ltd. in UK and KK Dnaform in Japan have commercial license, we have referred these companies in the Table of Materials. We have modified and added the custom order information in **Line 131**. We would like to show the SINEUPs ordering web page of Cell Guidance systems Ltd. in the video.

In case of lines 207-213 (RNA extraction, DNase treatment, and cDNA synthesis part), and 226-229 (Imaging analysis), we have added more details to the protocol to make it more comprehensive and user-friendly. Please refer to **Lines 216-252** (**Step 5 and 6**) and **266-278** (**Step 7**) in the revised version.

16. Lines 147-148: What volume of transfection reagent is used? Please specify the incubation temperature.

**Response:**

We have addressed this comment in **Lines 141-143** as “After 24 h, transfect 4 µg of SINEUPs for a 6 well-plate or 800 ng of SINEUPs for a 24 well-plate with transfection reagent (10 µL in 6 well-plate and 3 µL in 24 well-plate) (see **Table of Materials**), and incubate at 37 °C in a 0.5 % CO2 incubator for 24 h”.

17. Line 167: Please describe how to check protein concentration.

**Response:**

We are sorry for the insufficient explanation in this part. We have added the description of protein concentration check protocol in **Step 3.4** (from **Lines 162-180**).

18. Lines 186-190: Please describe how hybridization is done.

**Response:**

The hybridization protocol is already stated in these lines. Our explanation might be confusing, so we have slightly rephrased this in **Lines 196-200** as-

**“**In the case of SINEUP-GFP, hybridize the protein with anti-GFP antibody (diluted 1:2000 in blocking solution) (see **Table of Materials**) by incubating the membrane for 30 min with shaking at room temperature. As an internal control protein, detect β-actin protein by hybridizing with Anti-β-Actin antibody (diluted 1:2000 in blocking solution) (see **Table of Materials**) for 30 min with shaking at room temperature.**”**

19. Line 191: What volume of TBST buffer is used to wash?

**Response:**

We have modified the phrase as “Add 1 X TBST buffer (137 mM NaCl, 20 mM Tris-HCl (pH 7.6), 0.1 % Tween-20) to the container until the membrane is completely soaked and wash for 5 min at room temperature. Repeat the wash step two more times (a total of three washes).” in Lines **201-203**.

20. Line 200-201: Please add more details here.

**Response:**

We have modified this part in **Step 4.9 (Lines 210-214)** as “Mix equal volume of HRP-Enhanced Chemi Luminescence (ECL) detection reagent 1 and 2 (see **Table of Materials**). Transfer the membrane to 2 mL ECL reagent mix, cover the box with aluminum foil and let it incubate for 1-2 min at room temperature. Carefully remove the membrane from ECL reagent mix and expose using a luminescence imaging instrument.”

21. Please ensure that conditions and primers are listed all PCR procedures.

**Response:**

We are sorry for not being clear about some of the PCR conditions and primer details. We have now mentioned the details of cDNA synthesis and qRT-PCR in **Step 6 (Lines 238-264)**.

22. Please include single-line spaces between all paragraphs, headings, steps, etc.

**Response:**

We have formatted the text to include single-line spacing where needed.

23. After you have made all the recommended changes to your protocol (listed above), 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.

**Response:**

We have revised the protocol and highlighted the **Steps 1, 2, 3, 4, and 7** which we find crucial to convey the message of this protocol and suitable for the video.

24. 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.

**Response:**

We have made changes in the highlighted part as per the suggestion.

25. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

**Response:**

Following the editor’s comment, this time we have paid close attention to highlighting and made sure that it fits JoVE guidelines.

26. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:  
a) Critical steps within the protocol  
b) Any modifications and troubleshooting of the technique  
c) Any limitations of the technique  
d) The significance with respect to existing methods  
e) Any future applications of the technique

**Response:**

We thank the editor for this comment. As per the suggestion, we have extensively revised the Discussion to cover the following topics with proper citations:

**a) Critical steps within the protocol**

We have addressed this from **Lines 340-368** as “Designing an optimum BD is crucial to ensure SINEUP specificity and potency (extent of protein up-regulation). Previously, we screened 17 BDs of SINEUP-GFP by Western-blot analysis15 and found that the optimum BD overlaps the AUG-KOZAK sequence of GFP mRNA, though it may not be the case with other mRNAs and should be verified for each case. Another independent group also screened the BD using a different method31. As screening many BDs can be quite time-consuming and cumbersome, we introduced a high-throughput SINEUP detection method here. This method measures relative changes in GFP integrated density in SINEUP transfected-cells compared to the control vector transfected-cells. To ensure that cells in a particular well of a culture plate are transfected equally and GFP signal is not concentrated to only a certain region of the well, it is very important to distribute the cells equally in the wells in step 2.1. For this purpose, gently shake the plate 10 times back and forth (↑↓) after seeding the cells inside a clean bench and repeat in the 5 % CO2 incubator before starting the incubation.

Another critical step is the calculation of protein concentration in step 3.4. Miscalculations here can lead to the loading of an erroneous amount of protein during Western-blot analysis, consequently preventing detection of small changes in protein expression by some of the weak SINEUPs or generating false positives from overloading. It is recommended to freshly prepare the protein standard curve every time, making sure that equal amounts of standards and protein samples are measured in step 3.4.3. The protocol described here focuses on SINEUP-GFP, however Western-blot analysis can be used for any target mRNA of interest. The incubation time and concentration of antibodies should be optimized for each target to get the best result.

One of the unique features of SINEUPs is that target-mRNA expression level remains unaffected. It is important to treat RNA with DNase to avoid detection of transfected SINEUPs and genomic DNA by qRT-PCR. SINEUP RNA and target mRNA expression should be measured by qRT-PCR to confirm the success of both transfection and SINEUP activity. SINEUPs contain a SINE sequence, which is abundant throughout both the human and mouse genomes. To avoid non-specific detection of SINE sequences, it is not recommended to design qRT-PCR primers to the SINE sequence.”

**b) Any modifications and troubleshooting of the technique**

We have covered this part in **Lines 369-379** as “In this protocol we used human cell lines, but SINEUPs are efficacious in a number of cell lines from several different species12-14,18,19. The cell culture and transfection conditions can be modified according to different cell lines as long as these maintain transfection efficiency of the SINEUP vectors. Moreover, alternative methods of RNA extraction, cDNA synthesis and protein concentration checking can be employed, given that they preserve the required RNA and protein quality for qRT-PCR and Western-blot analysis. 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. It is to be noted that if the distribution of cells and transfection is equal throughout a well then it is not necessary to scan the whole well for GFP fluorescence: half or one-quarter of the area of a well might be enough to discern SINEUP effect depending on experimental skills.”

**c) Any limitations of the technique**

We have addressed this topic from **Lines 403-406** stating- “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.”

**d) The significance with respect to existing methods**

We have discussed this topic from **Lines 390-402** as “This screening protocol is very fast. We do not need to fix and collect cells, but just need to place the living cell culture plate in the imaging instrument. We propose to use this high-throughput screening protocol to select optimum BDs of SINEUPs, and evaluate potential candidates by Western-blot analysis. Thus, selected candidates with optimum BDs can be applied to increase antibody production18,19,21. Currently, the RNA therapeutic field is dramatically growing. For instance, siRNA, ASO, mRNA and CRISPR RNA therapies, are widely employed to control mRNA expression of their respective targets9,32. In this context, SINEUPs are in their infancy, but so far none of the studied SINEUPs changed expression of target mRNAs. In addition, SINEUPs do not edit target mRNA, but only up-regulate translation of mRNA. Furthermore, loss-of-function diseases resulting from haploinsufficiency can be targeted by SINEUP therapy, achieving a 2-fold induction of the deficient protein17,33. Although off-target effects need to be further studied, SINEUPs potentially and specifically target a single, expressed mRNA with a complementary sequence to the BD.”

**e) Any future applications of the technique**

We have discussed future applications of SINEUPs in **Lines 380-389** as “Establishing a high-throughput SINEUP detection protocol allows for simultaneous screening of multiple BDs targeting a given mRNA in cultured cells. This is important as the rules governing optimum targeting by the BD are still unclear. Such a multiplex screening system allows for large-scale testing of many SINEUPs against different genes, useful for targeting multiple genes involved in a particular signaling pathway for instance. 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 addition, we summarized the applications in **Lines 407-409** as “Nevertheless, SINEUPs can be applied to gain-of-function studies, to enhance antibody production, and as an RNA therapy to up-regulate expression of deficient proteins within the range of 1.5- to 3.0-fold.”

27. References: Please do not abbreviate journal titles.

**Response:**

We have modified references to include full journal titles.

28. Table of Equipment and Materials: Please remove trademark (™) and registered (®) symbols. Please provide lot numbers and RRIDs of antibodies, if available.

**Response:**

We have edited the Table of Materials to accommodate the recommended changes.