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To:
Dr. Alisha DSouza
Senior Review Editor of JoVE

Yokohama, August 4, 2018

We hereby submit the revised manuscript entitled “Cell based assays of SINEUP non-coding RNAs that can specifically enhance mRNA translation” by Hazuki Takahashi, Harshita Sharma and Piero Carninci to be considered for publication as original methods article of JoVE produced Video.

In summary, we have identified a novel class of antisense RNAs, which have the function of positively regulate protein translation (Carrieri et al, Nature, 2012, doi: 10.1038/nature11508), likely be enhancing the interaction of the sense RNA with the translational machinery mediated by a SINEB2 element embedded in the transcript. We named these antisense RNAs “SINEUPs”, since they use a SINE element to UP-regulate translation.

Following the initial discovery, in this manuscript we show the cell based assay methods of the SINEUPs translation-enhancing protein activity by high throughput micro well image cytometer, to produce synthetic SINEUPs against a broad range of targets. Synthetic SINEUPs are being used in many studies to specifically increase protein translations, as a counterpart of siRNAs. Here, we explore the design of the antisense regions (called “binding domain”) to produce a first milestone work towards optimization of the design of these RNAs, which is needed to broadly expand applications for many users to enhance protein translation.

The revised version of the manuscript address all of the comments raised by the editor and reviewers, as outlined in the rebuttal letter.

In addition to this, we would like to disclose all the potential conflict of interest around this study. In particular, Piero Carninci (PC) is inventors in a patent (US9353370B2 and patents applications related to this patent, as EU and Japanese patents) owned by our main employers, our academic research institutions (RIKEN). PC funded TransSINE Technologies, Inc., a company located in Japan with the mission to develop and commercialize products based on of the US9353370B2 and related patents. This includes the commercialization of SINEUPs in plasmid expression vectors, including basic vectors and custom made constructs (see details from the UK representative web site: http://www.cellgs.com/services/sineup.html). We believe that association with this company to provide reagents worldwide will help to further enable colleagues to use the technology.

TransSINE Technologies has not provided actual funding to this research, nor direct salaries or any direct benefits to any of the members participating in this study. TransSINE Technologies has only provided information that was necessary to the design and the execution of the project. PC, who is also affiliated to TransSINE Technologies, has a key role in the design of the study. TransSINE Technologies has not influenced any conclusions of the study. Affiliation of PC to TransSINE Technologies does not alter our adherence to JoVE policies on sharing data and materials.

We believe that the revised manuscript satisfies all the requirements for the publication in JoVE. We are looking forward to hearing from you.

Sincerely,
Dr. Piero Carninci

TITLE:

Cell Based Assays of SINEUP Non-coding RNAs that can Specifically Enhance mRNA Translation

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KEYWORDS:

SINEUPs, antisense RNA, long noncoding RNAs (lncRNAs), translation regulation, high-throughput screening, RNA therapy, antibody production

SUMMARY:

SINEUPs are synthetic antisense non-coding RNAs, which contain a binding domain (BD) and an effector domain (ED) and up-regulate translation of target mRNA. Here, we describe detection methods for SINEUPs in cultured cell lines, analysis of their translation-promoting activity by Western-blot and a semi-automated high throughput imaging system.

ABSTRACT:

Targeted-protein enhancement is of importance not only for the study of biological processes but also for therapeutic and biotechnological applications. Here, we present a method to selectively up-regulate protein expression of desired genes in cultured cells by means of synthetic antisense non-coding RNAs known as SINEUPs. This positive control of gene expression is at the post-transcriptional level and exerted by an inverted short interspersed nuclear element (SINE) repeat at the 3' end of SINEUPs that comprises its effector domain (ED). SINEUPs can specifically bind to any protein-coding mRNA of choice through its binding domain (BD), a region designed to complement the sequence within the 5' untranslated region (5' UTR) and around the start codon of the mRNA. Target-specific SINEUPs designed in this manner are transfected to cultured cells, and protein and RNA are extracted for downstream analyses, generally 24-48 h post-transfection. SINEUP-induced protein up-regulation is detected by Western-blot analysis and RNA expression is measured using real-time quantitative reverse transcription PCR. We have

observed that BD design is critical for achieving optimum SINEUP activity and that testing different BD sizes and positions with respect to the start codon of the target mRNA is recommended. Therefore, we describe here a semi-automated high-throughput imaging method based on fluorescence detection that can be implemented to target mRNA fused with green fluorescent protein (GFP). SINEUPs specifically enhance translation within normal physiological range of the cell, without altering the target transcript level. This method has been successfully employed against a range of endogenous and exogenous targets, in a wide variety of human, mouse, and insect cell lines along with *in vivo* systems. Moreover, SINEUPs have been reported to increase antibody production and work as an RNA therapeutic against haploinsufficient genes. The versatile and modular nature of SINEUPs makes them a suitable tool for gene-specific translational control.

INTRODUCTION:

In the post-genomic era, many insights have been gained into the gene regulatory roles of non-coding antisense transcripts owing to the development of next-generation sequencing technologies[1-3] and gene-editing tools. This category of transcripts, which was previously considered to be "transcriptional trash", is now established as a key player of genetic regulation. Antisense transcripts are reported to modulate chromatin and control stability and expression of their cognate protein-coding sense mRNA[4,5]. In most cases, this mode of regulation is negative and antisense transcripts silence their sense counterparts through RNA-RNA interactions[6,7]. This trait of natural antisense transcripts has been utilized to downregulate desired genes in the form of synthetic small interfering RNA (siRNA), microRNA (miRNA), and antisense oligos (ASO)[8-11] leaving a technological void for targeted gene up-regulation.

One intriguing study changed the perspective of the antisense RNA field by demonstrating that antisense long non-coding RNAs (AS lncRNAs) of the genes *Uchl1* (ubiquitin C-terminal hydrolase L1) and *Uxt* (ubiquitously-expressed transcript) positively regulate translation of their cognate sense mRNA at the post-transcriptional level in mice[12]. The 5′ end of these AS lncRNAs overlaps with several bases in the 5′ untranslated region (5′ UTR) of their corresponding sense transcripts, and the non-overlapping 3′ end contains an inverted repeat of a retrotransposon belonging to the short interspersed nuclear element (SINE) family. Interestingly, we found that the main driving force behind this translational up-regulation is the embedded SINE repeat and it is not limited to mouse SINE repeats only. Human Alu repeat-containing AS lncRNAs also enhanced translation of target sense mRNAs, reinforcing the idea of a novel class of SINE-driven regulatory antisense non-coding RNAs, appropriately named SINEUPs[13]. Recent studies showed that the potential of natural SINEUPs is preserved in synthetic SINEUPs designed to specifically target various endogenous and exogenous genes[14,15]. SINEUPs have two important features: first the "binding domain" (BD) which is generally the 5′ end region complementary to the sequence encompassing the primary start codon of a protein-coding mRNA, and second the "effector domain" (ED) as it incorporates an inverted repeat of SINE which is a prerequisite for SINEUP function[14] (**Figure 1**). SINEUPs can be customized to design a BD specifically targeting a gene of choice. This can then be harnessed to dissect the function of a particular gene involved in a biological pathway, as a better alternative to a conventional mRNA overexpression strategy. In

addition, this versatile tool can be applied to boost antibody production, and as a drug to treat haploinsufficient diseases caused by insufficient dosages of functional proteins[16-21].

The advantages of SINEUP technology are manifold. It does not alter the expression of the target at the transcriptional level. Longer BDs provide more specificity to SINEUPs, while not inducing a dsRNA-dependent stress response[15]. The protein induction is maintained within the normal physiological range of the cell, preventing any deleterious effect due to aberrant or excessive protein expression. SINEUPs are compatible with a wide variety of mouse, human, and hamster cultured cell lines, for instance, HEK293T/17, HepG2, HeLa, CHO, MN9D, and many more[12-15,18,19]. SINEUPs can efficiently target endogenous genes, transiently overexpressed genes, and FLAG-tagged or luciferase-fusion genes, eliminating the need of gene-specific antibodies[14,18]. The basic SINEUP analysis requires routine cell culture, transfection, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), real time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) instruments and settings, and expertise can be gained in a short time.

Here, we describe a method for targeting genes with synthetic SINEUPs to up-regulate translation, an effect contrary to other technologies such as RNA interference[9] and ASO gapmers[11,22,23]. A widely-used method for RNA-guided gene activation is clustered regularly interspaced short palindromic repeats-based activation (CRISPRa), where the gene expression is triggered at the transcriptional level[24]. This method, though simple and fast, requires multiple single guide RNAs (sgRNAs) targeting the same gene for higher efficiency, thereby increasing the chances of off-target binding[25]. In addition, the key enzyme of the CRISPRa system, the catalytically dead CRISPR-associated protein 9 (dCas9) has low binding specificity and sgRNAs targeting bi-directional promoter regions can non-specifically up-regulate nearby genes[25]. Conversely, SINEUPs bind to target mRNA at a single binding region with high specificity and do not affect expression of nearby genes. We discuss the basic steps involved in SINEUP design, transfection, protein and RNA expression analyses. Furthermore, we present a semi-automated, high-throughput imaging system to screen multiple SINEUPs at once, which is useful for detection of optimum SINEUPs.

PROTOCOL:

1. Design of BDs of SINEUP Constructs for Target mRNAs

1.1. Check transcription starting site (TSS) and translation starting site of target mRNAs in the cells of interest. Acquire TSS data from both the ENCODE and FANTOM projects (cap analysis of gene expression, CAGE) (ZENBU: http://fantom.gsc.riken.jp/zenbu/).

1.2. Open the browser, search genes of interest and check TSS by CAGE peaks.

1.3. Consult the example analysis of cell and tissue specific TSS of Parkinson disease protein 7 (*PARK7*) mRNA shown in **Figure 2**.

1.4. Design BD sequences of several different lengths corresponding to 40 bases upstream and 32 bases downstream of the first methionine (AUG), 72 nt in total.

1.5. Custom order the designed BDs in SINEUP expression vector (see **Table of Materials**).

Note: Check the sequence specificity by basic local alignment search tool (BLAST) to avoid off-target effects[26].

2. Cell Culture and SINEUP Transfection

2.1. Seed cells of interest in a 6 well-plate or 24 well-plate for 24 h before transfection of SINEUPs. In the case of human embryonic kidney cell line (HEK293T/17) (see **Table of Materials**), seed 0.5 x 10^6 cells per well of a 6 well-plate or 1.5 x 10^5 cells per well of a poly-D-lysine coated 24 well-plate (see **Table of Materials**). It becomes 70% confluent after 24 h.

2.2. 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 5% CO_2 incubator for 24 h. For analyzing SINEUP-GFP, transfect 0.6 µg of pEGFP-C2 (see **Table of Materials**) and 3.4 µg of SINEUP-GFP in one well of a 6 well-plate, or 130 ng of pEGFP-C2 and 670 ng of SINEUP-GFP in one well of a 24 well-plate.

2.3. Wash cells with 10 mL of phosphate buffer saline (PBS) (37 mM NaCl, 8 mM Na_2HPO_4, 2.6 mM KCl and 1.5 mM KH_2PO_4) and add 2 mL of PBS. Harvest 3/4 of cells (1.5 mL) from 1 well for protein extraction (go to protocol 3 for protein extraction) and 1/4 of cells (0.5 mL) for RNA extraction by centrifugation at 6,000 x g for 5 min at 4 °C (go to step 5 for RNA extraction).

Note: Analyze the effect of SINEUP-GFP in a poly-D-lysine coated 24 well-plate by imaging. Go to step 7 (imaging analysis).

3. Protein Extraction

3.1. Carefully aspirate 1.5 mL of PBS and add 140 µL of lysis solution (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% (w/v) Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 µg/mL leupeptin, and 0.005% (w/v) phenylmethylsulfonyl fluoride (PMSF)) to the cells (see **Table of Materials**).

3.2. Mix by pipetting and then mix thoroughly by rotating at slow speed for 1 h at 4 °C. Collect supernatant by centrifugation at 14,000 x g for 10 min at 4 °C.

3.3. Check protein concentration by colorimetric assay (see **Table of Materials**). Prepare all the reactions at room temperature.

3.3.1. Prepare 5-6 times dilution of bovine serum albumin (BSA) protein standard in ultrapure water with concentrations ranging from 0.2-1.5 mg/mL protein. Prepare fresh standards each time.

3.3.2. Prepare working reagent A' by mixing 1 mL of reagent A (alkaline copper tartrate solution) with 20 µL of reagent S (surfactant solution).

3.3.3. Load 5 µL of water (negative control), BSA standard (protein standard and positive control) or protein sample in each well of a 96-well plate.

3.3.4. Add 25 µL of reagent A' in each well. Carefully add 200 µL of reagent B (Folin reagent) per well avoiding any bubble formation. Cover the plate with aluminum foil and wait for 5-10 min.

3.3.5. Measure the protein absorbance at 750 nm with a spectrophotometer. The absorbance is stable for at least 1 h.

3.3.6. Prepare standard curve by plotting BSA standard protein concentrations (mg/mL) on the x-axis and their respective absorbance on the y-axis. Calculate sample protein concentration by applying standard curve equation.

Note: Pause the protocol at this step if needed or go to step 4. For long-term storage, snap freeze protein samples in liquid nitrogen and store at -80 °C.

4. Protein Separation by SDS-PAGE and Detection of Target Proteins with Antibodies (Western-blot Analysis)

4.1. Add one volume of 2x loading dye (0.1 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 1.42% 2-mercaptethanol and 0.2% bromophenol blue) to each volume of protein sample from step 3.3. Heat at 90 °C for 5 min and immediately cool on ice for 1 min.

4.2. Load 10-20 µg of protein samples to 10% SDS poly-acrylamide gel and separate at 100-150 V) (see **Table of Materials**).

4.3. Transfer protein from gel to 0.45-µm nitrocellulose membrane (see **Table of Materials**) by semi-dry transfer instrument with transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol) at 25 V for 30 min.

4.4. Add blocking solution (5% non-fat dry milk in 1x TBST buffer (137 mM NaCl, 20 mM Tris-HCl (pH 7.6), 0.1% Tween-20)) (see **Table of Materials**) to the container until the membrane is completely soaked and incubate it at room temperature for 30 min.

4.5. 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.

4.6. Add 1x TBST buffer 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).

4.7. Hybridize GFP protein to diluted (1:1000 in blocking solution) anti-rabbit antibody conjugated with horseradish peroxidase (HRP), and hybridize β-actin protein to diluted (1:1000 in blocking solution) anti-mouse antibody conjugated with HRP on the membrane for 30 min with shaking at room temperature.

4.8. Wash membrane with 1x TBST buffer for 5 min at room temperature. Repeat the wash step two more times (a total of three washes).

4.9. Mix equal volume of HRP-enhanced chemiluminescence (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.

5. RNA Extraction and DNase Treatment

5.1. Extract total RNA following the standard protocol for RNA extraction from cells grown in a monolayer[27] or alternatively use a commercially available RNA extraction kit (see **Table of Materials**). To state in brief, add any monophasic lysis reagent (MLR) of guanidine isothiocyanate and phenol to the harvested cells in step 2.3 (1 mL MLR per ~5 million cells)[27].

Note: Change gloves frequently. Use RNase-free reagents, and diethylpyrocarbonate (DEPC)-treated plastic ware and glassware to prevent RNA degradation. Pause the protocol at this step if needed. Store extracted RNA at -80 °C.

5.2. Add 0.1 volume of 10x DNase buffer and 2 U of DNase to the purified RNA from step 5.1 (see **Table of Materials**).

5.3. Set the reaction volume to 50 µL with nuclease-free water and mix gently. Incubate at 37 °C for 30 min.

5.4. Add 0.1 volume of re-suspended DNase inactivation reagent and mix well (see **Table of Materials**). Incubate for 5 min at room temperature with gentle shaking.

5.5. Centrifuge at 10,000 x g for 90 s. Transfer the supernatant (DNA-free RNA) to a fresh RNase-free 1.5 mL tube. Be careful not to touch the pellet because this can cause carry-over of DNase which is troublesome for downstream steps.

5.6. Quantitate RNA by measuring A_{260} and A_{260}/A_{280} ratio (for pure RNA, the range is 1.8-2.0) in a UV spectrophotometer.

6. First Strand cDNA Synthesis and qRT-PCR Analysis

6.1. Perform first strand cDNA synthesis following a standard cDNA synthesis protocol as below (see **Table of Materials**).

6.1.1. Mix 0.75 µM oligo dT primer, 4.3 µM random primer, dNTPs (10 mM each) with 200-500 ng total RNA (from steps 5-8) in 10 µL of total reaction volume. Incubate at 65 °C for 5 min and then immediately put on ice.

6.1.2. Mix 1x reverse transcriptase buffer, 1 U of RNase inhibitor, 10 U of reverse transcriptase, and add to 10 µL RNA-primer mix from 6.1.1.

6.1.3. Set the total reaction volume to 20 µL with nuclease-free water if required. Mix gently and incubate the reaction mix at 30 °C for 10 min, then at 42 °C for 60 min, and finally at 95 °C for 5 min to inactivate the enzyme. Cool the tubes on ice.

Note: Thaw RNA samples and all the reagents on ice and prepare the reaction mix on ice. If target RNAs are only polyA RNAs, use oligo dT primer only (2.5 µM). Pause the protocol if needed. Store synthesized cDNA at -20 °C.

6.2. Perform qRT-PCR in technical triplicates (n=3, C_T standard deviation > 0.2) and within biological replicates (n ≥ 3) using 1 µL of 10 ng cDNA, 0.4 µL of reverse primer (10 µM), 0.4 µL of forward primer (10 µM), 10 µL of mixture solution of DNA polymerase, 0.4 µL of double-strand DNA staining dye, 10 µL of nuclease-free water (reaction volume was 20 µL) to detect PCR products using following conditions; hold stage (1 cycle) for 30 s at 95 °C, cycling stage (40 cycles) for 5 s at 95 °C and for 30 s at 60 °C, melt curve stage. Primer examples are for Human Gapdh_Fw: TCTCTGCTCCTCCTGTTC, Human Gapdh_Rv: GCCCAATACGACCAAATCC, EGFP_Fw: GCCCGACAACCACTACCTGAG, EGFP_Rv: CGGCGGTCACGAACTCCAG, SINEUP-GFP_Fw: CTGGTGTGTATTATCTCTTATG and SINEUP-GFP_Rv: CTCCCGAGTCTCTGTAGC. See **Table of Materials**. Analyze quantitative RNA expression with $2^{-\Delta\Delta Ct} \pm$ SD method[28].

7. Imaging Analysis of SINEUPs by Semi-automated Detection Method

7.1. Wash cells with 0.5 mL of PBS.

7.2. Add 2.0 µg of Hoechst 33342 (see **Table of Materials**) (dissolving in 500 µL of PBS) to each well of the 24-well plate and incubate cells at 37 °C for 20 min to stain the nucleus.

7.3. Measure Hoechst stained cells to count the total number of cells at a blue emission maximum of 461 nm, and intensity of green fluorescence to count number of GFP positive cells at a green emission maximum of 510 nm by using a high-throughput micro-well image cytometer

(see **Table of Materials**). Analyze protein up-regulatory effect of the SINEUPs by calculating GFP integrated intensity, which is the sum of all pixel intensities displaying signal in segmented objects calculated for each channel, using Imaging software[29].

7.4. Harvest cells to check RNA expression (back to steps 5 and 6).

REPRESENTATIVE RESULTS:

SINEUP-GFP is a synthetic SINEUP containing both an optimum BD (-28/+4 overlap to GFP mRNA) and an ED (inverted SINE B2 from AS-*Uchl1*) that can up-regulate GFP mRNA translation (**Figure 3A and 3B**) without changing the expression of GFP mRNA (**Figure 3C and 3D**). To screen optimum BDs and EDs for SINEUPs, we developed a protocol of semi-automated image analysis that improved detection time and increased the number of samples being simultaneously screened compared with a conventional Western-blot analysis[15]. As shown in **Figure 4**, this high-throughput imaging system took 3 days (Western-blot analysis took 2 weeks for 48 SINEUPs). 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. Nevertheless, we detected significantly higher levels of GFP fluorescence compared with the control (**Figure 5A and 5B**).

FIGURE AND TABLE LEGENDS:

Figure 1: Basic design of the synthetic SINEUPs. BD: binding domain to target mRNA, ED: effector domain containing an inverted SINE sequence.

Figure 2: Transcription starting sites (TSSs) of human Parkinson disease protein 7 (*PARK7*) mRNA in specific tissues and cells as detected by CAGE analysis. A. A snapshot showing a TSS search result for human *PARK7* mRNA using an omics data integration and interactive visualization system[30]. The horizontal green arrow in the Entrez gene hg19 track indicates the genomic position of the reference human *PARK7* mRNA and the vertical green bars in the FANTOM5 CAGE 1 and 2 tracks indicate the sum of TSSs (measured as transcripts per million (tpm)) from 1,829 types of tissues and cells. **B.** Zoom in of the marked region of the TSS in panel A (1/354 scale: 35.4 kb to 100 bp). Grey shaded area in the FANTOM5 CAGE phase 1 and 2 tracks indicates the TSS of 6 specific cells out of the total 1,829, which are listed at the bottom of the figure. The numbers (11.369, 4.998, 4.304, 3.509, 3.477, and 3.055) corresponding to these listed cells indicate transcripts per million for each specific cell at the grey shaded positions of the TSS.

Figure 3: Western-blot analysis confirming up-regulation of translation by SINEUP-GFP. A. SINEUP-GFP was examined by Western-blot with anti-GFP antibody. **B.** Results were normalized to the intensity of the β-actin protein band. (**C**) GFP mRNA and (**D**) SINEUP RNAs expression were detected by qRT-PCR. ***$p < 0.0005$, n=3, two-tailed student's t-test, error bars are standard deviation. This figure has been modified from Takahashi *et al.*[15].

Figure 4: Schematic of SINEUP semi-automated image analysis. Day 1: Seed cells of interest in a 24 well-plate. Day 2: Transfect GFP and SINEUP-GFP. Day 3: Analyze the effect of SINEUPs by measuring GFP integrated intensity.

Figure 5: High-throughput analysis of translation up-regulation by SINEUP-GFP. A. Comparison of GFP fluorescence between control and SINEUP-GFP transfected cells[15]. Scale bar = 2 mm. **B.** GFP integrated intensity was normalized to the total cell number counted by nuclear staining with Hoechst 33342. ***p < 0.0005, n=3, two-tailed student's t-test, error bars are standard deviation. FOV: field of view. This figure has been modified from Takahashi *et al.*[15].

DISCUSSION:

We described here a protocol to specifically enhance protein production of a target mRNA by means of a SINE-containing non-coding RNA, called SINEUPs. As a representative example, optimum synthetic SINEUP-GFP is shown to up-regulate the translation of GFP mRNA 2.6-fold as measured by Western-blot analysis.

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 analysis[15] 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 method[31]. 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% CO_2 incubator before starting the incubation.

Another critical step is the calculation of protein concentration in step 3.3. 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 3.3.3. The protocol described here focuses on SINEUP-GFP, but 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 RNAs 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

391 detection of SINE sequences, it is not recommended to design qRT-PCR primers to the SINE
392 sequence.
393
394 In this protocol we used human cell lines, but SINEUPs are efficacious in a number of cell lines
395 from several different species[12-14,18,19]. The cell culture and transfection conditions can be
396 modified according to different cell lines as long as these maintain transfection efficiency of the
397 SINEUP vectors. Moreover, alternative methods of RNA extraction, cDNA synthesis, and protein
398 concentration checking can be employed, given that they preserve the required RNA and protein
399 quality for qRT-PCR and Western-blot analysis. While we used a specific high-throughput micro-
400 well cytometer, which enabled detection of GFP fluorescence across the entire well, other
401 cytometers with a similar detection range can be used[31]. It is to be noted that if the distribution
402 of cells and transfection are equal throughout a well, then it is not necessary to scan the whole
403 well for GFP fluorescence: half or one-quarter of the area of a well might be enough to discern
404 SINEUP effect depending on experimental skills.
405
406 Establishing a high-throughput SINEUP detection protocol allows for simultaneous screening of
407 multiple BDs targeting a given mRNA in cultured cells. This is important as the rules governing
408 optimum targeting by the BD are still unclear. Such a multiplex screening system allows for large-
409 scale testing of many SINEUPs against different genes, useful for targeting multiple genes
410 involved in a particular signaling pathway for instance. Furthermore, it can be utilized to expand
411 the search for effective SINEUPs targeting several mRNAs, designing different SINEUP BDs around
412 AUG-Kozak region (see **Figure 1**), co-transfecting full length target mRNAs (5' UTR-CDS-3' UTR)
413 fused with GFP mRNA in cultured cells to find the optimum SINEUPs, and subsequently testing
414 BD candidates against endogenous mRNA in cultured cells and *in vivo* model animals, from
415 humans and mice to other animal and plant species.
416
417 This screening protocol is very fast. We do not need to fix and collect cells, but just need to place
418 the living cell culture plate in the imaging instrument. We propose to use this high-throughput
419 screening protocol to select optimum BDs of SINEUPs and evaluate potential candidates by
420 Western-blot analysis. Thus, selected candidates with optimum BDs can be applied to increase
421 antibody production[18,19,21]. Currently, the RNA therapeutic field is dramatically growing. For
422 instance, siRNA, ASO, mRNA, and CRISPR RNA therapies, are widely employed to control mRNA
423 expression of their respective targets[9,32]. In this context, SINEUPs are in their infancy, but so far
424 none of the studied SINEUPs changed expression of target mRNAs. In addition, SINEUPs do not
425 edit target mRNA, but only up-regulate translation of mRNA. Furthermore, loss-of-function
426 diseases resulting from haploinsufficiency can be targeted by SINEUP therapy, achieving a 2-fold
427 induction of the deficient protein[17,33]. Although off-target effects need to be further studied,
428 SINEUPs potentially and specifically target a single, expressed mRNA with a complementary
429 sequence to the BD.
430
431 A limitation of this high-throughput protocol is that it is not suitable to screen BDs of SINEUPs in
432 *in vivo* mouse models because the protocol measures the GFP integrated intensity only. As
433 SINEUPs are natural antisense lncRNAs that act post-transcriptionally, they cannot be applied
434 when the target mRNA is missing in the cells or tissue samples.

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 1.5-3.0-fold. The methods described here present a useful guide to target and detect SINEUP-induced translation enhancement of desired mRNA and provide a new tool for post-transcriptional gene regulation.

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DISCLOSURES:

The corresponding author Piero Carninci is one of the founders of TransSINE Technologies Co. Ltd. that holds intellectual property on filed patent (EP2691522A4 and JP2017169573A), and granted patent (US9353370B2) for SINEUP technology.

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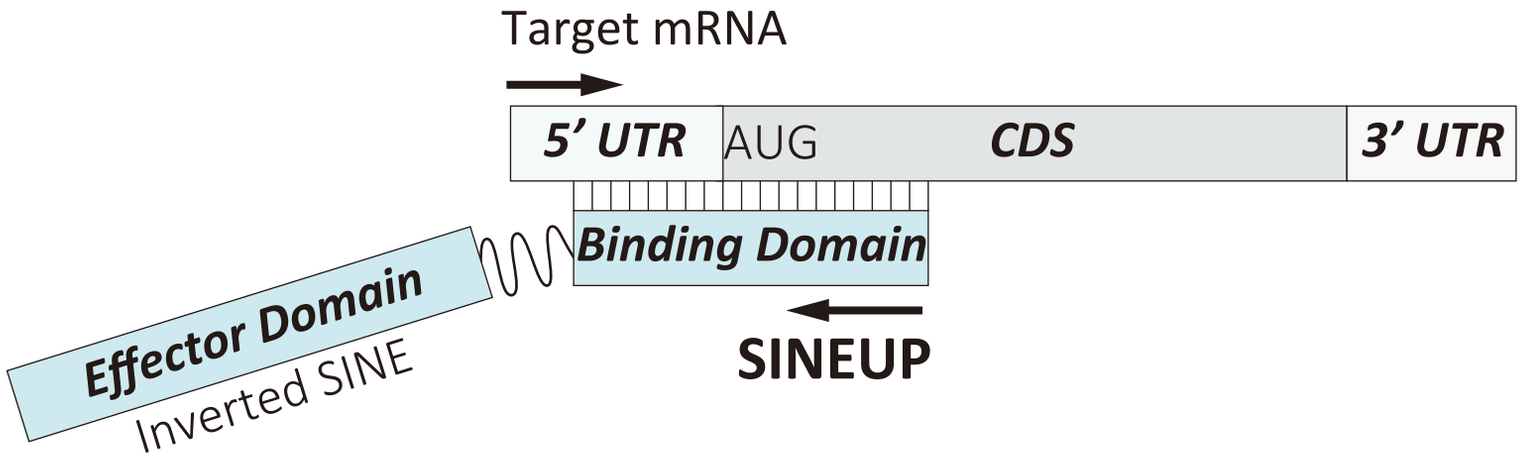

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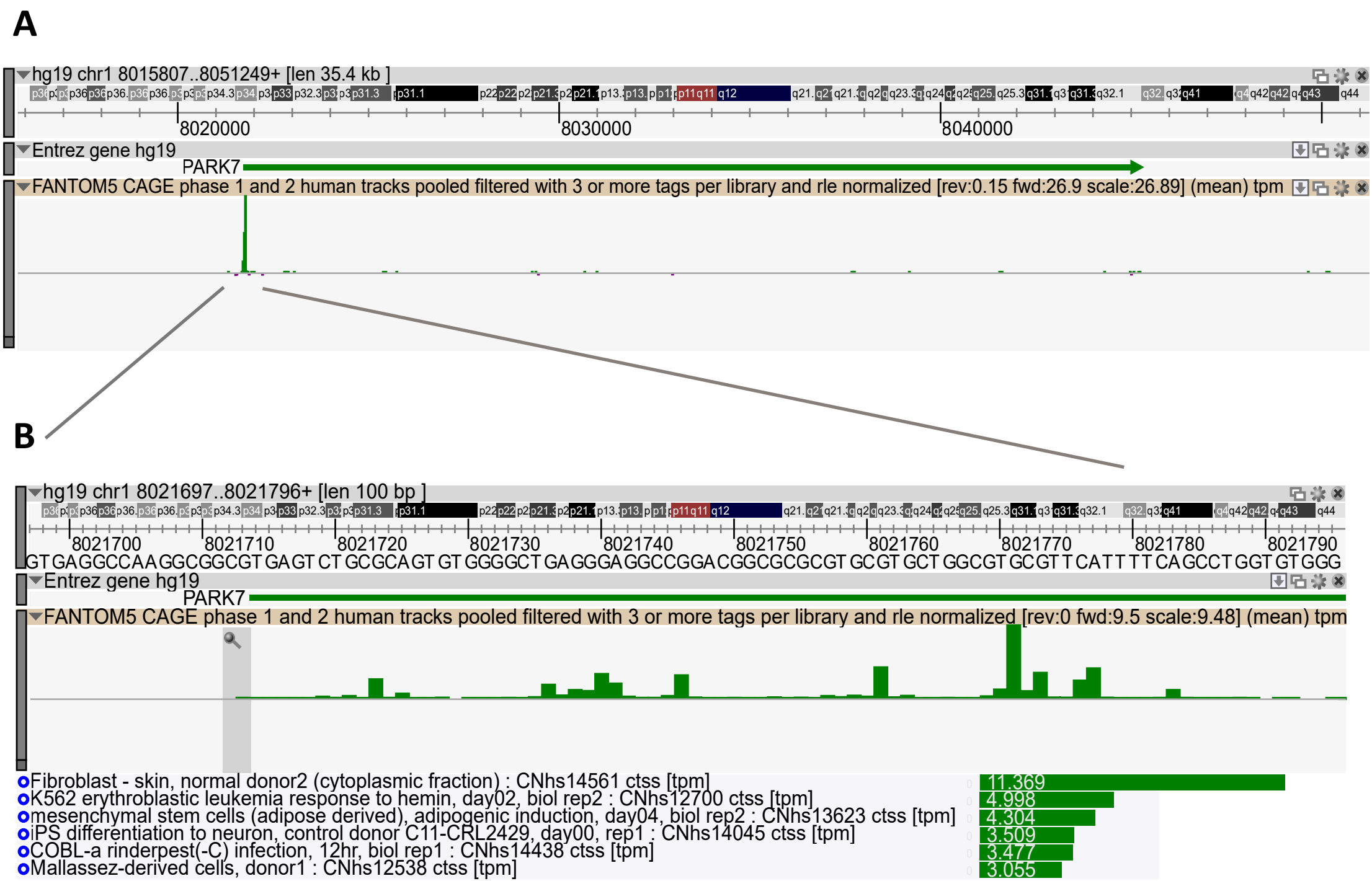

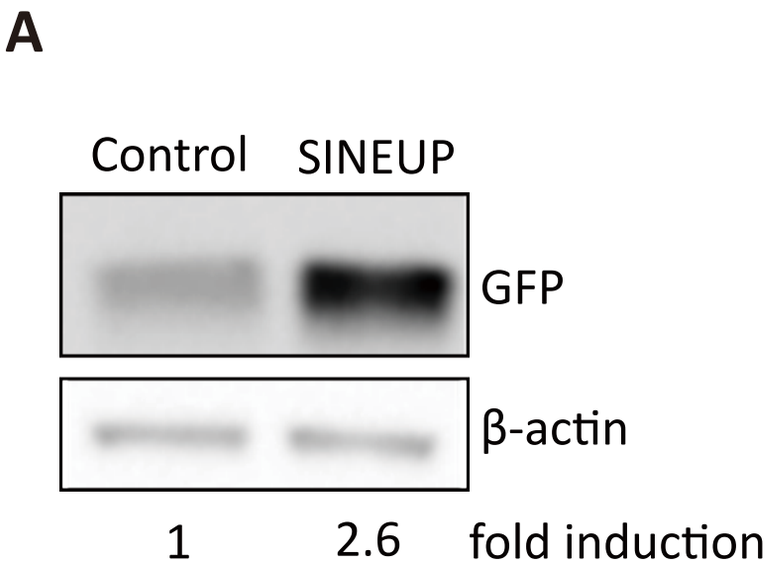

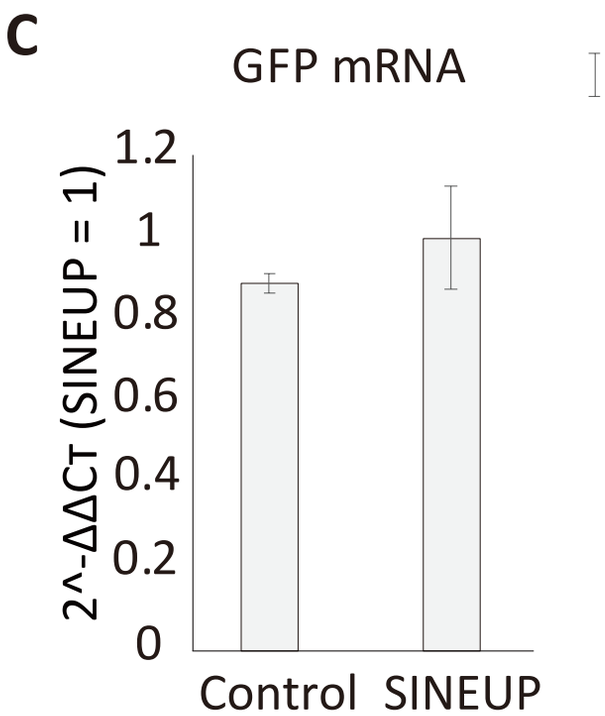

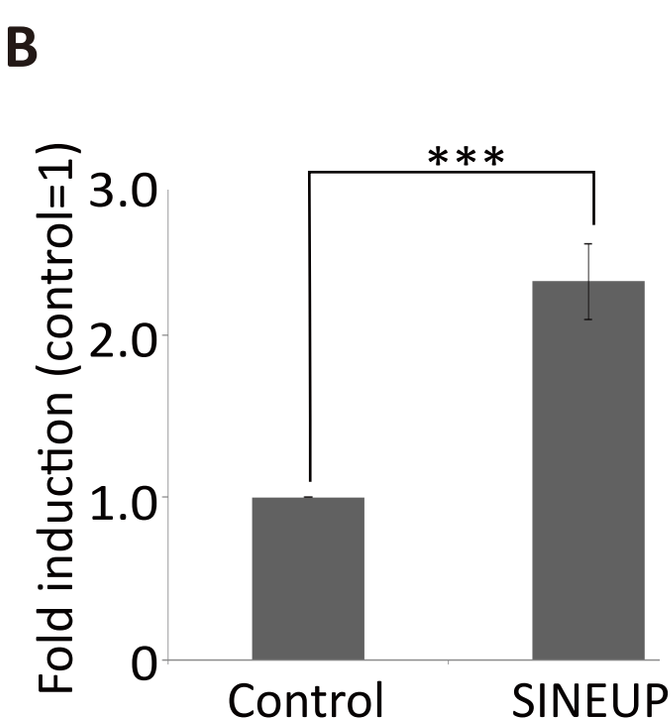

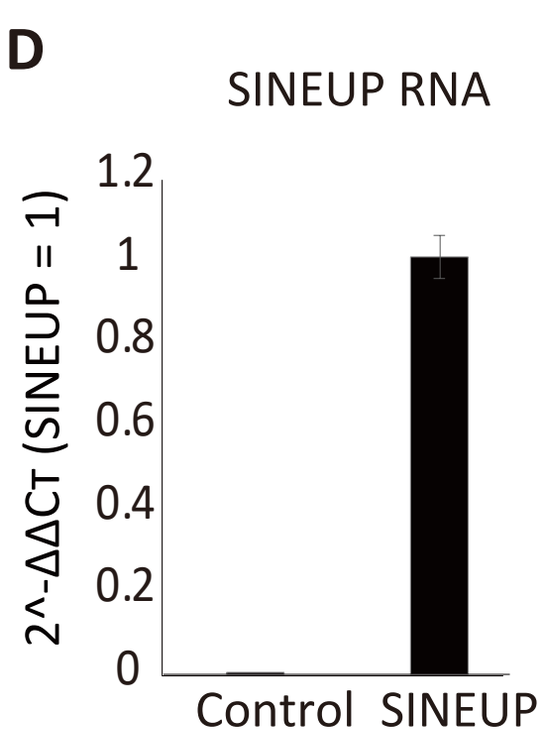

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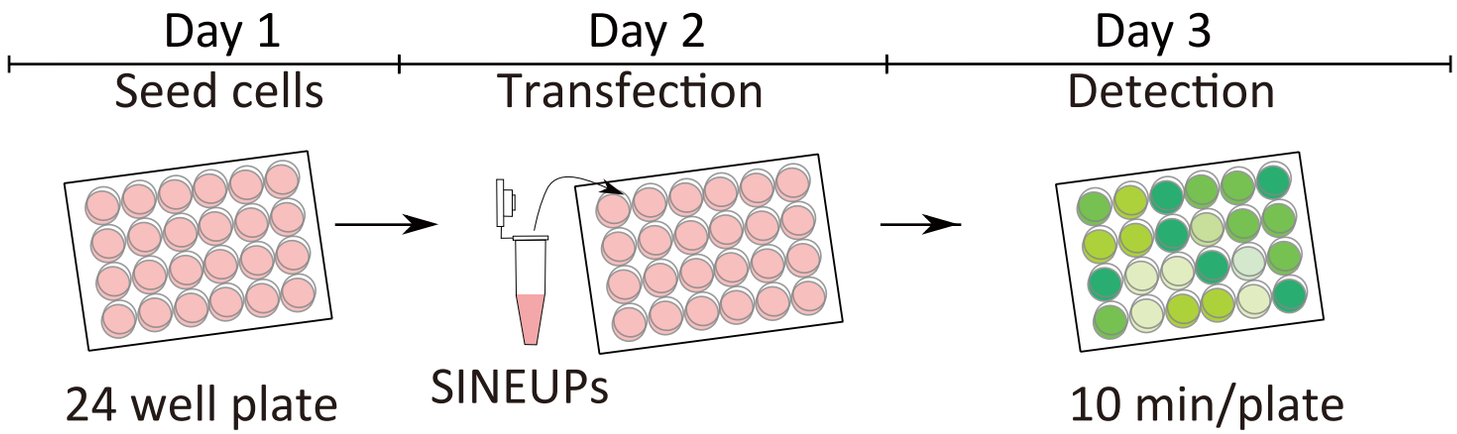

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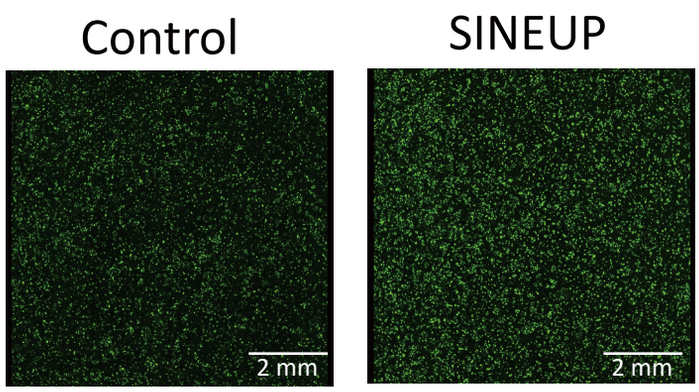

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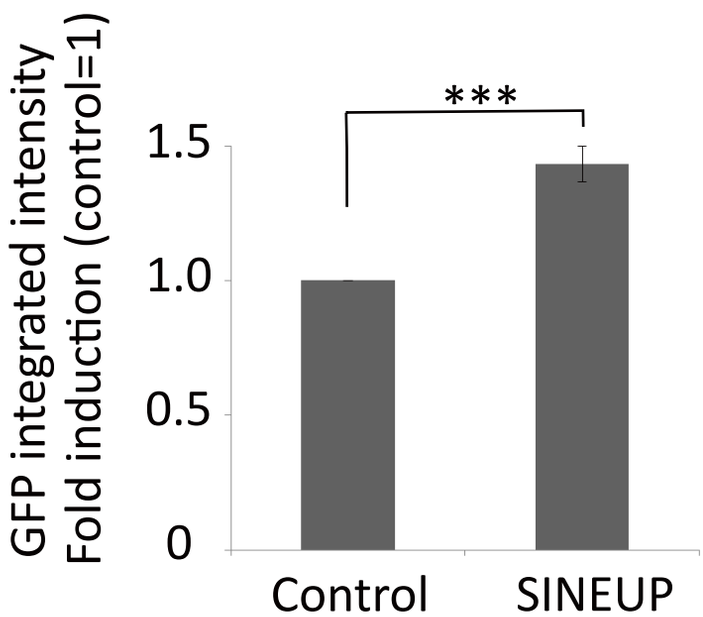

Name of Material/ Equipment

Synthetic SINEUP

HEK293T/17
Falcon Multiwell Plate For Cell Culture 6 well plate
Corning BioCoat Poly-D-Lysine 24 well Plate
Lipofectamine 2000 Transfection Reagent
pEGFP-C2
Cell Lysis Buffer (10x)
PMSF
DC protein assay kit II
10% Mini-PROTEAN TGX Precast Protein Gels, 12-well, 20 µl
Amersham Protran Premium NC 0.45 （150 mm×4 m）
Nonfat Dry Milk
GFP Tag Antibody
Monoclonal Anti-β-Actin antibody produced in mouse
Polyclonal Goat Anti Rabbit Immunoglobulins
Polyclonal Goat Anti mouse Immunoglobulins
ECL Western Blotting Detection Reagents
Maxwell Rapid Sample Concentrator (RSC) Instrument
Maxwell RSC simplyRNA Cells Kit
TURBO DNA-free Kit
PrimeScrip 1st strand cDNA Synthesis Kit
TB Green Premix Ex Taq II
Hoechst3342
Celigo S

Company	Catalog Number
Cell Guidance Systems Ltd. in UK and K.K.DNAFORM in Japan	https://www.cellgs.com/items/sineupand8482.html and https://www.dnaform.jp/en/information/20130320
ATCC	ATCC CRL-11268
Corning	6902A01
Corning	08-774-124
Thermo Fisher Scientific	11668027
Clontech	#6083-1
Cell Signaling Technology	#9803S
Cell Signaling Technology	#8553S
BIO RAD	#5000112JA
BIO RAD	#4561035
Amasham	10600013
Cell Signaling Technology	#9999S
Thermo Fisher Scientific	A-6455
SIGMA	A5441
Dako	P0448
Dako	P0447
Amersham	RPN2109
Promega	AS4500
Promega	AS1390
ambion	AM1907
TaKaRa	6110A
TaKaRa	RR820A
Thermo Fisher Scientific	H3570
Nexcelom Bioscience	200-BFFL-S

Comments/Description

Step 1.5.

Step 2.1.
Step 2.1.
Step 2.1.
Step 2.2.
Step 2.2.
Step 3.1. This is pre-mixed cell lysis solution.
Step 3.1. To complete cell lysis buffer, add 0.005% (w/v) PMSF to 1 x cell lysis buffer.
Step 3.3.
Step 4.2.
Step 4.3., This is a 0.45 µm nitrocellulose membrane.
Step 4.4. A component of the blocking solution.
Step 4.5. Lot number: 1495850, RRID: AB_221570
Step 4.5. Lot number: 026M4780V, RRID: AB_476744
Step 4.5. This is secondary antibody conjugating with HRP for anti GFP antibody. Lot number: 2001752
Step 4.5. This is secondary antibody conjugating with HRP for anti β-Actin antibody. Lot number: 20019
Step 4.9.
Step 5.1. This is commercially available RNA exreaction instrument.
Step 5.1. This is commercially available RNA exreaction kit.
Step 5.2 and 5.4. The kit contains DNase, DNase buffer and inactivation reagent.
Step 6.1. The kit contains reagents of first strand cDNA synthesis.
Step 6.2.
Step 7.2.
Step 7.3. This is a high throughput micro-well image cytometer.

5, RRID:AB_2617138
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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): e0183229[15]). 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 % CO_2 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 analysis[15] 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 method[31]. 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 % CO_2 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 species[12-14,18,19]. 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 used[31]. 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 production[18,19,21]. 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 targets[9,32]. 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 protein[17,33]. 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 (5' UTR-CDS-3' 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.

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 method[31]."
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 used[31]."

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 (5' UTR-CDS-3' 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.

To:

Dr. Alisha DSouza

Senior Review Editor of JoVE

Yokohama, August 4, 2018

We have submitted a manuscript entitled “Cell based assays of SINEUP non-coding RNAs that can specifically enhance mRNA translation” by Hazuki Takahashi, Harshita Sharma and Piero Carninci to reuse Figure 3 and Figure 5 from a previous publication of PLOS journal. [Takahashi, H. et al. Identification of functional features of synthetic SINEUPs, antisense lncRNAs that specifically enhance protein translation. PLOSONE. 13 (2), e0183229, doi:10.1371/journal.pone.0183229, (2018).]

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Sincerely,

Dr. Piero Carninci