User Guide

SINEUP™ Kit

Gene Knock-up System

Cat GE02

Protocol Version 1.1 **NOTE Commercial Use of this product requires a license.**

Contents

Storage

Upon receipt, store all components at -20°C.

Product Components

- 10 µg pDUAL/eGFP plasmid, desiccated pellet, 1 vial.
- 10 µg pDUAL/eGFP_eGFP-BD/ED plasmid, desiccated pellet, 1 vial.
- 3 µg pDUAL/ED plasmid, desiccated pellet, 1 vial.

Product Overview

SINEUP™ technology enables the targeted up-regulation of an endogenous or co-transfected gene in a wide range of cell lines. A complete SINEUP™ construct consists of an Effector Domain (ED) combined with a target gene-specific Binding Domain (BD).

The SINEUP™ gene knock-up technology typically allows 2 to 5-fold and up to 10-fold increases in expression levels of target proteins by increasing the efficiency of translation. Transcription of the target gene and levels of its mRNA are unaltered. As with RNAi, which can be used to knockdown (down-regulate) protein levels, SINEUP™ technology does not rely on the direct manipulation of the target gene. It is not a gene editing technique.

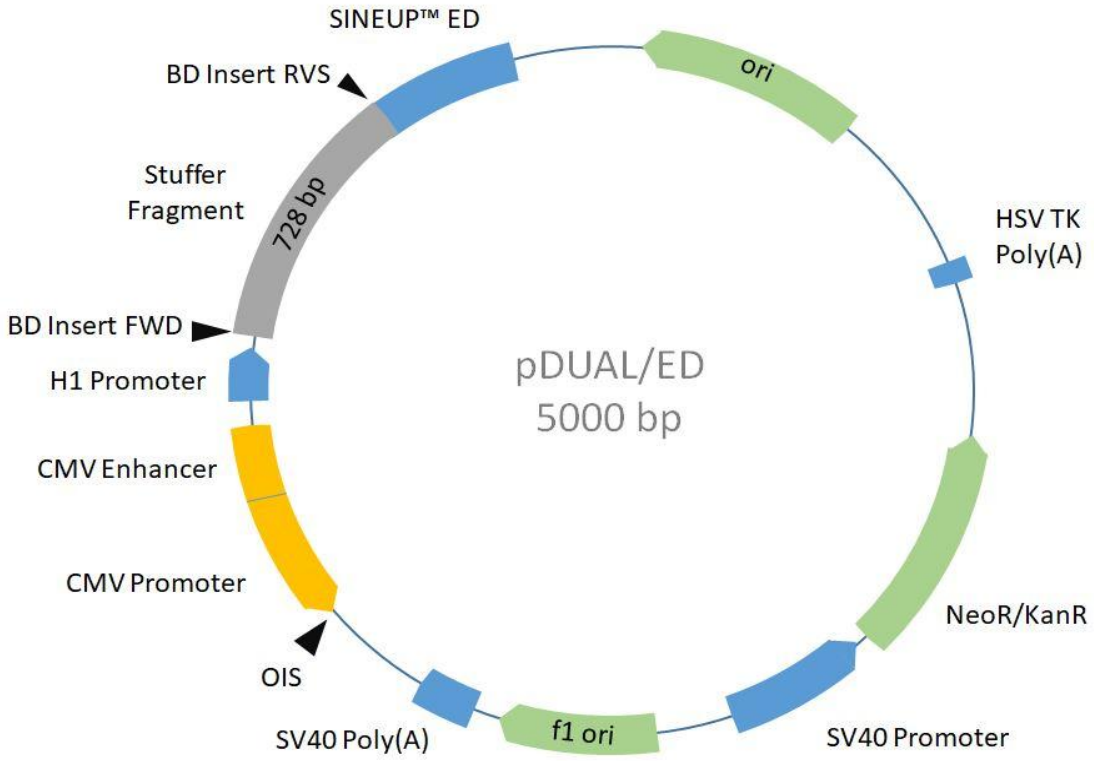
The SINEUP™ Gene Knock-up kit contains a pair of plasmids which act as a positive control. The first plasmid of the pair is pDUAL/eGFP, which expresses an enhanced GFP protein downstream of a CMV promoter. The second plasmid is pDUAL/eGFP_GFP-BD/ED, which expresses eGFP protein downstream of a CMV promoter and also an eGFP-BD, followed by the SINEUP™ ED downstream of an H1 promoter. This pair of vectors can be used to transfect cells of interest and evaluate the difference in expressed eGFP protein and eGFP mRNA levels.

The third vector, pDUAL/ED, contains the SINEUP™ ED, upstream of which the BD for any target gene can be cloned in via specific restriction sites. Once the BD has been inserted, the complete SINEUP™ will be downstream of the H1 promoter.

Subsequent to generation of the desired SINEUP™ construct, mammalian cells of interest can be transfected using commonly used transfection methods. The amount of expressed protein from the transfected vs. non-transfected cells can be assessed by western blot. mRNA levels can be measured using quantitative RT-PCR. Both data sets combined can be analyzed to determine the extent of the gene knock-up and to confirm the mechanism is through increased translation efficiency and not increased transcription.

Vector Information

pDUAL/ED plasmid map



Vector components

Component	Description
SINEUP™ ED	SINEUP™ Effector Domain, forms a secondary structure which recruits ribosomes to the mRNA and therefore increases translation efficiency.
BD Insert RVS	The 3' site of insertion for the target gene Binding Domain. EcoRI restriction site.
Stuffer Fragment	728 bp fragment which is to be replaced by the target gene Binding Domain. Allows colony PCR checkpoint subsequent to transformation with the Binding Domain of interest.
BD Insert FWD	The 5' site of insertion for the target gene Binding Domain. XhoI restriction site.
H1 Promoter	Provides competent *in vitro* transcription.
CMV Enhancer	Human cytomegalovirus (CMV) immediate early enhancer.
CMV Promoter	Human cytomegalovirus (CMV) immediate early promoter. Provides competent *in vitro* transcription in the anti-sense orientation.
OIS (Optional Insert Site)	Contains a SalI restriction site at the 5' end and a BsrGI restriction site at the 3' end. Offers optional insertion site for gene of interest in the anti-sense orientation.
SV40 Poly(A)	Provides effective transcription termination and polyadenylation of mRNA.
f1 ori	f1 bacteriophage origin of replication.
SV40 Promoter	Enables high-level expression of the neomycin/kanamycin resistance gene.
Neo/Kan resistance	Gene for neomycin phosphotransferase in mammalian cells allows stable clone selection with G418/ Gene for kanamycin resistance to select for transformants in *E. coli*.
HSV TK Poly(A)	Herpes simplex virus thymidine kinase polyadenylation signal.
ori	High-copy-number origin of replication.

Partial sequence information

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CCATTTACCG TAAGTTATGT AACGCGGAAC TCCATATATG GGCTATGAAC TAATGACCCC 60
GTAATTGATT ACTATTAATC TCACTAAAGA TATTTGCATG TCGCTATGTG TTCTGGGAAA 120
TCACCATAAA CGTGAAATGT CTTTGGATTT GGGAATCTTA TAAGTTCTGT ATGAGACCAC 180
TCACGCGTGA GCTCGGTACC CTCGAGCCGC GCGTGACCTG CAAATCTTTG GCCTCGATCT 240
GCTTGTCCTT GATGGCAACG ATGCGTTCAA TAAACTCTTG TTTTTTAACA AGTTCCTCGG 300
TTTTTTGCGC CACCACCGCT TGCAGCGCGT TTGTGTGCTC GGTGAATGTC GCAATCAGCT 360
TAGTCACCAA CTGTTTGCTC TCCTCCTCCC GTTGTTTGAT CGCGGGATCG TACTTGCCGG 420
TGCAGAGCAC TTGAGGAATT ACTTCTTCTA AAAGCCATTC TTGTAATTCT ATGGCGTAAG 480
GCAATTTGGA CTTCATAATC AGCTGAATCA CGCCGGATTT AGTAATGAGC ACTGTATGCG 540
GCTGCAAATA CAGCGGGTCG CCCCTTTTCA CGACGCTGTT AGAGGTAGGG CCCCCATTTT 600
GGATGGTCTG CTCAAATAAC GATTTGTATT TATTGTCTAC ATGAACACGT ATAGCTTTAT 660
CACAAACTGT ATATTTTAAA CTGTTAGCGA CGTCCTTGGC CACGAACCGG ACCTGTTGGT 720
CGCGCTCTAG CACGTACCGC AGGTTGAACG TATCTTCTCC AAATTTAAAT TCTCCAATTT 780
TAACGCGAGC CATTTTGATA CACGTGTGTC GATTTTGCAA CAACTATTGT TTTTTAACGC 840
AAACTAAACT TATTGTGGTA AGCAATAATT AAATATGGGG GAACATGCGC CGCTACAACA 900
CTCGTCGTTA TGAACGCAGA CGGGCAATTC CAGTGCTAGA GGAGGTCAGA AGAGGGCATT 960
GGATCCCCCA GAACTGGAGT TATACGGTAA CCTCGTGGTG GTTGTGAACC ACCATGTGGA 1020
TGGATATTGA GTTCCAAACA CTGGTCCTGT GCAAGAGCAT CCAGTGCTCT TAAGTGCTGA 1080
GCCATCTCTT TAGCTCCAGT CTCTTAAGCT TTCTAGAGGA TCTGGAATAA ATTAATAACT 1140
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Features: pDUAL/ED Seq FWD primer binding site; H1 promoter; Forward Colony PCR primer binding site; XhoI site; Stuffer Fragment; EcoRI site; SINEUP™ ED; Reverse Colony PCR primer binding site

Guidelines for use

The SINEUP™ ED is supplied as an insert cloned into the pDUAL vector. Excision of the entire Stuffer Fragment can be achieved using XhoI and EcoRI. To target a gene for knock-up of protein expression, a BD sequence complementary to the 5' untranslated region (UTR) and first several bases downstream of the initiation methionine of the target gene is required. This BD should be synthesized and inserted in place of the Stuffer Fragment. The cloning site for BD insertion is XhoI and EcoRI. The BD should correspond to the antisense sequence immediately surrounding the initiation codon of the target gene. The nucleotide positions upstream of the AUG are referred to as negative and the nucleotides downstream of the AUG are referred to as positive. +1 is A in AUG. Recommended BD sequence designs are typically -40/+32; -40/+4 and -14/+4. It was empirically found that any of these BD designs work for most genes so it is highly recommended to try all three of the above designs first. Further optimization by changing the length and/or positioning of the BD may provide additional improvements to knock-up of protein expression. Please note that translation of the SINEUP™ RNA does not occur. Therefore the BD does not have to be in-frame with the ED. A discussion of BD design is provided in reference 2.

Protocol

Positive Control Experiment

Before proceeding with generation of the SINEUP™ fragment for the gene of interest, it is strongly recommend to test the cell line in question with the provided positive control which consists of the pDUAL/eGFP and pDUAL/eGFP_eGFP-BD/ED plasmid pair. The test is performed by transfecting cells with each of the plasmids and subsequently analyzing the levels of expressed eGFP using a fluorescence plate reader or western blot. The levels of eGFP mRNA should be measured by RT-qPCR.
Comparing these results, the mechanism of SINEUP™ as well as the degree of gene knock-up can be evaluated: cells transfected with pDUAL/eGFP_eGFP-BD/ED should exhibit elevated levels of protein expression compared to cells transfected with pDUAL/eGFP while maintaining the same eGFP mRNA levels. Previous tests suggest that all commonly used transfection methods and transfection reagents are suitable. For both control plasmids, 10 µg are provided each, enabling at least 5 separate positive control experiments.

A. Binding Domain Design

1. Locate the 5' sequence of the gene of interest and identify the sequence complementary to the 5' UTR and immediately after the initiation methionine.

3. It is recommended to design -40/+32; -40/+4 and -14/+4 (+1 is A in AUG) as Binding Domains (BD), see 'Guidelines For Use' section.

4. Each BD should be flanked by an XhoI restriction site at the 5' end.

5. Each BD should be flanked by a 10 nt long linker followed by an EcoRI restriction site at the 3' end. The linker can contain any 10 nt of choice.

6. The resulting BD sequences should be synthesized by the method of choice.

For a detailed guide describing how to design Binding Domains, please refer to the 'Binding Domain Design' Technical Note available on our website.

B. Cloning of Construct

1. Re-suspend vectors in TE buffer (pH 8.0), briefly centrifuge vials before opening. Digest the pDUAL/ED vector with XhoI/EcoRI. The kit provides 3 µg of the vector.

2. Once digested, proceed with gel purifying the resulting 4,276 bp linearized fragment.

3. Perform ligation reactions for the 4,276 bp linearized fragment with each of the three linear Binding Domains obtained in section A. It is recommended to use a 1:10 molar ratio of vector:insert.

C. Transformation and Sequence Verification

1. Transform the ligation reaction into *E. coli* cells. Please see vector map on page 5 for selective reagents details.

2. Once single colonies are generated, sequence 3 colonies for each construct in order to identify the colony that contains the correct sequence. Primer for sequencing is pDUAL/ED Seq FWD: 5'- GTAAGTTATGTAACGCGGAAC -3'. The binding site for this forward primer is located upstream of the H1 promoter (see 'features' on page 5).

Optional: An additional checkpoint for confirming correct sequence can be introduced by performing colony PCR on colonies generated at the first step of this section.

The PCR can be performed with the following primer pair: Forward colony PCR primer: 5'- TTGCATGTCGCTATGTGTTCTGGGAAATCACC -3'; Reverse colony PCR primer: 5'- GGAACTCAATATCCATCCACATGGTGGTTCACAAC -3'.

This procedure enables to discriminate between colonies that contain the initial Stuffer Fragment of 728 bp and the much smaller (<100 bp) Binding Domain insert.

The forward PCR primer has a binding site which ends 76 bp upstream of the Stuffer Fragment/BD insert (in the H1 promoter). The reverse PCR primer has a binding site which ends 71 bp downstream of the Stuffer Fragment/BD insert (in the SINEUP Effector Domain).

Once the SINEUP™ constructs of interest are generated, verified and purified, proceed to transfect the cells of interest using the method of choice.

D. Transfection

1. Transfect the cells of interest with the plasmid generated in section C. Any common transfection method/reagent can be used.

2. For each transfection replicate it is recommended to culture cells on an approximately 9 cm^2 surface area (one well of a 6-well TC plate).

Additional Product Notes

In order to assess the efficiency of the SINEUP™ in any given cell line, test cells transfected with the Binding Domain designed for the gene of interest by measuring the levels of expressed protein against non-transfected cells. Also, measure the mRNA levels for the gene of interest. As stated above, the levels of mRNA for the gene of interest in both transfected and non-transfected cells should be similar.

If the expression of the gene of interest is exogenous, the Binding Domain construct can be co-transfected with a vector expressing said gene of interest. Alternatively, the Optional Insert Site (OIS) of the pDUAL/ED plasmid can be utilized (see vector map on page 5-6). The OIS allows to clone the gene of interest downstream of the CMV enhancer+promoter on the pDUAL/ED plasmid using the SalI and BsrGI sites.

Technical questions may be addressed to tech@cellgs.com

References:

1. Long non-coding antisense RNA controls Uchl1 translation through an embedded SINEB2 repeat, Carrieri C., *et al*., *Nature* (2012), 491, 454-457.
2. SINEUPs: A new class of natural and synthetic antisense long non-coding RNAs that activate translation. Zucchelli et al., S. RNA Biol. (2015);12:771-9.

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