Luciferase-based siRNA Target Test Kit (25x)
Catalog # N0080

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I. General Purpose

Luciferase-based siRNA Target Test Kit is designed to provide a quantitative approach for evaluation of sequence potential to serve as an efficient target for RNA interference (RNAi). Current computer-based RNAi target searching algorithms are not perfect, leaving the probability of selected sequence to be a good target from 30% to 60%. It is therefore clear that the ability of computer-selected sequence to induce RNA interference has to be confirmed experimentally. The **siRNA Target Test Kit** allows for screening a large number of potential siRNA target sequences in simple transient transfection experiments, measuring the reduction in reporter (firefly Luciferase from *Photinus pyralis*) gene expression.

In most commercially available vectors and kits intended for the same purpose, the gene silencing potential of a target sequence is tested on the fused transcript bearing both reporter gene and gene of interest. In such systems some unpredictable factors like chimeric mRNA folding and target accessibility may affect the observation of RNAi. Also the distance between the target site and reporter gene coding region along the mutual messenger RNA plays a role in the reporter gene silencing: even if mRNA is cleaved at the point of siRNA target, translation of the reporter gene can still go on till the RNA degradation by nonspecific nucleases reaches the reporter gene coding region. Such kind of effects may obscure the real efficiency of particular targets.

Unlike other kits, the **siRNA Target Test Kit** measures the efficiency of target sequences *per se*. The short target sequences are located at defined positions immediately upstream or downstream of the reporter gene coding region. Independent evaluation of the reporter gene silencing for two different positions of the same target permits to avoid or reduce the influence of target accessibility factor and other factors related to RNA conformation, thus making comparison between various targets more reliable. Another advantage of the **siRNA Target Test Kit** is that it makes possible evaluation of the siRNA target potential even in those cases when the gene of interest is not available as full-size cDNA clone.

II. How the System Works

1. Description

The **siRNA Target Test Kit** consists mainly of three vectors: one effector plasmid (psiRNA) producing double-stranded siRNA, and two reporter plasmids (psiTEST-target-LUC and psiTEST-LUC-target) expressing Luciferase reporter gene fused to siRNA target sequence (Fig. 1). The system design is based on the fact that the nucleotide sequence of siRNA is identical to the sequence of corresponding siRNA target. Thus the same short synthetic DNA fragment has to be cloned into all three plasmids, giving rise to siRNA coding sequence in effector plasmid and to the siRNA target sequence in both reporter plasmids.

siRNA expression from the effector plasmid psiRNA is driven by dual promoter expression cassette bearing human U6 and H1 small nuclear RNA promoters in opposite orientation to each other. Such dual promoter constructs were shown to efficiently express double-stranded siRNA molecules (1-2) which could be directly accepted by RNA Interference Searching Complex (RISC), the multi-enzyme complex with RNase activity specifically digesting messenger RNA at the siRNA target site (3). This permits to avoid the stage of dicer treatment which is necessary for hairpin siRNA producing vectors to convert single-stranded hairpin shRNA to double-stranded siRNA.

Detailed structure of the double-promoter siRNA expressing cassette is shown in Fig. 2. siRNA coding sequence N_1-N_{19} is placed between the U6 and H1 promoters. RNA synthesis driven by the U6 promoter starts from the nucleotide N_1 which has to be G, and terminates at the stretch of 5 T invading the body of H1 promoter. As a result, produced RNA has 2 or 3 uridines at its 3' terminus. Similarly, the RNA produced from the H1 promoter starts with the nucleotide N'_{19}, terminates at 5 T stretch at the beginning of the opposite U6 promoter, and also bears 2 or 3 uridines at its 3' terminus. Two halves of siRNA join together, forming the functional double-stranded siRNA with protruding 3'-termini.

A mixture of siRNA-producing effector plasmid and one of the target-bearing reporter plasmids has to be cotransfected transiently into mammalian cells. Measuring the reporter gene silencing due to the effect of RNA interference will give a quantitative evaluation of the siRNA target potential.

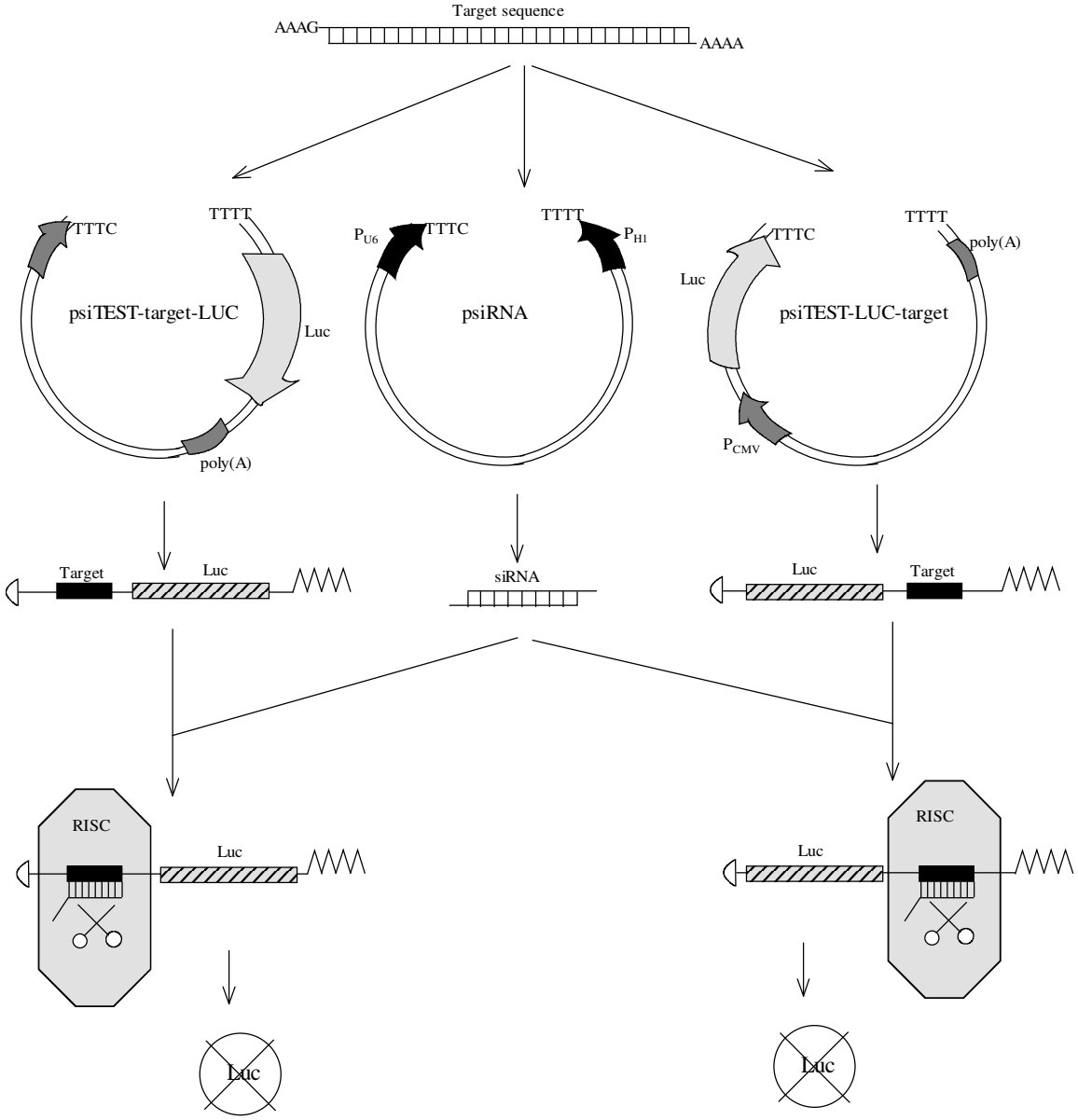

Fig. 1. Construction of effector and reporter plasmids and their mechanism of action.

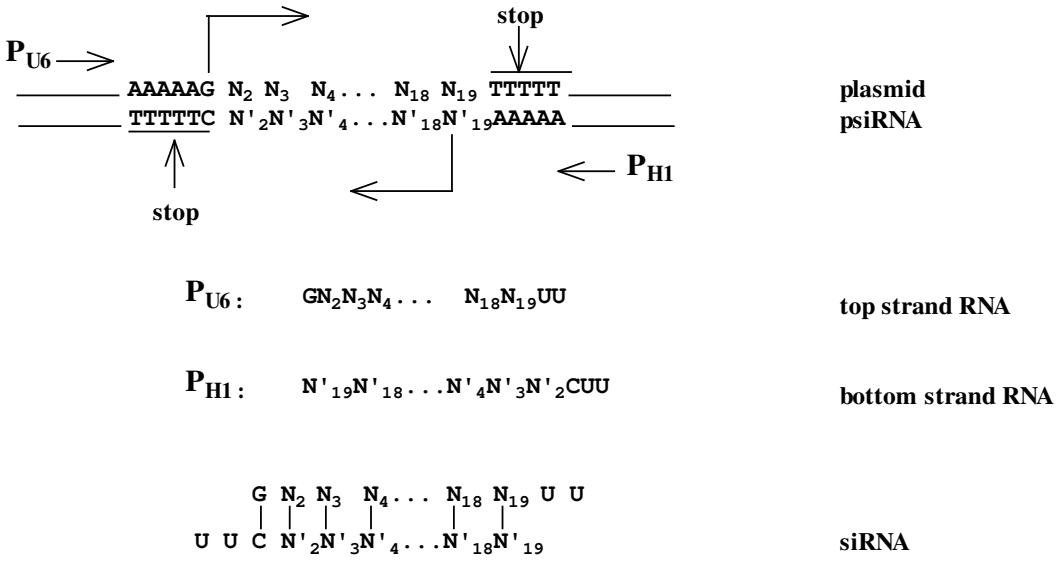

Fig. 2. siRNA expression from the dual promoter effector plasmid psiRNA.

2. Model experiment

To demonstrate the utility of **siRNA Target Test Kit**, 10 target sequences against Green Fluorescent Protein from copepoda *Pontellina plumata* (CopGFP) were chosen for the screening of their gene silencing potential. Corresponding oligonucleotides were synthesized and subcloned into linearized psiRNA, psiTEST-target-LUC, and psiTEST-LUC-target vectors. Then effector and reporter plasmid constructs were cotransfected into HEK-293 human cells in 24-well plates in duplicates, and Luciferase activity was measured 48 h post transfection (Fig. 3). Control psiRNA-negative effector plasmid expressing irrelevant siRNA was also cotransfected with each reporter target-bearing plasmid to give Luciferase activity reference point (100%).

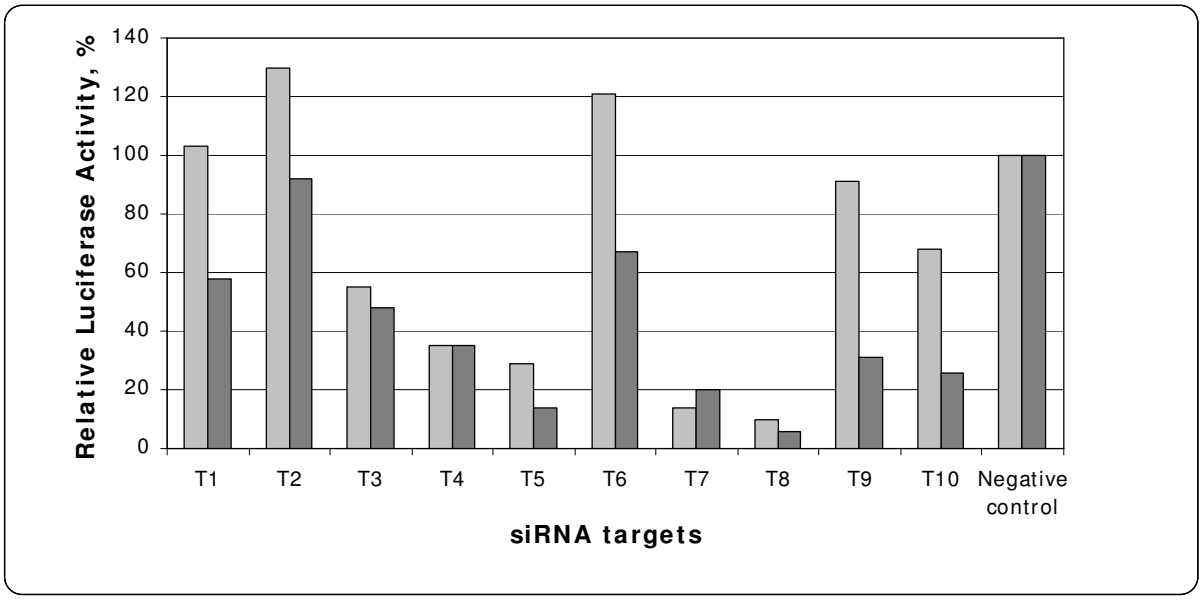

Fig. 3. Target efficiency determination by siRNA Target Test Kit. 10 target sequences (T1—T10) from CopGFP gene were tested. Cells HEK–293 were cotransfected with the mixtures of siRNA-producing effector plasmid and corresponding reporter target-bearing plasmid (2:1). Effector plasmid psiRNA-negative expressing irrelevant siRNA was used as a control. Luciferase activity was measured 48 h post transfection, and relative Luciferase activity was calculated for each reporter construct generated from psiTEST-target-LUC (light columns, target site is located upstream of the Luciferase coding region) and psiTEST-LUC-target (dark columns, target site is located downstream of the Luciferase coding region).

As shown in Fig. 3, three out of ten targets (T5, T7, and T8) demonstrate significant silencing potential (relative Luciferase activity less then 30%) when placed both upstream and downstream of the Luciferase coding region. Please note that both types of reporter plasmids gave consistent results with regard to the level of gene silencing.

III. List of Components

1	psiRNA	linearized by Bbs I	(20 ng/µl)	25 µl
2	psiTEST-target-LUC	linearized by BsmB I	(20 ng/µl)	25 µl
3	psiTEST-LUC-target	linearized by BsmB I	(20 ng/µl)	25 µl
4	psiRNA-negative	supercoiled	(500 ng/µl)	50 µl
5	psiRNA-siLUC	supercoiled	(500 ng/µl)	50 µl
6	psiTEST-control-LUC	supercoiled	(500 ng/µl)	25 µl
7	psiTEST-LUC-control	supercoiled	(500 ng/µl)	25 µl
8	si-p53 Sense Oligo		(10 µM)	25 µl
9	si-p53 Antisense Oligo		(10 µM)	25 µl
10	Sequence/PCR Forward Primer for psiRNA		(10 µM)	40 µl
11	Sequence/PCR Forward Primer for psiTEST-target-LUC		(10 µM)	40 µl
12	Sequence/PCR Forward Primer for psiTEST-LUC-target		(10 µM)	40 µl
13	Cloning Enzyme Mix*			80 µl
14	Cloning Enzyme 10X Reaction Buffer**			160 µl
15	Manual			

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* A mixture of T4 DNA ligase and T4 Polynucleotide Kinase.

** 10X Reaction Buffer composition: 400 mM Tris-HCl, pH 7.8;
100 mM $MgCl_2$;
5 mM EDTA;
50 mM DTT;
5 mM ATP.

IV. siRNA target testing

1. Selection of Target Sequences

Several algorithms for siRNA target sequence selection have been developed recently (4-5). We recommend the following siRNA design software available through internet free access, which gave consistent results in our hands:

Dharmacon si*DESIGN* Center : http://design.dharmacon.com ;

Whitehead Institute for Biomedical Research, siRNA Selection Program : http://jura.wi.mit.edu/siRNAext ;

siRNA Design Software : http://i.cs.hku.hk/~sirna/software/sirna.php ;

siDirect siRNA online design site : http://design.RNAi.jp .

The optimal length of siRNA target sequence is 19 bases. While longer sequences may elicit nonspecific interferon-dependent response (6-7), they also can be assayed for RNAi by **siRNA Target Test Kit** if their length does not exceed 30 bp.

Transcription from the U6 promoter starts from the nucleotide **G**. Thus the first nucleotide of siRNA target sequence has to be **G**. One may use other nucleotides at the first position, but this is less preferable. In that case additional **G-C** nucleotide pair absent in the target sequence would appear in the double-stranded siRNA in the course of transcription.

2. Design of Oligonucleotides

Once the siRNA target sequence is selected, two oligonucleotides should be synthesized and cloned into all three kit vectors. If the target sequence is 19 nucleotides long and starts from nucleotide **G** (recommended), it can be written as :

5' **G-N_2-N_3-N_4-…-N_{18}-N_{19}** 3' (messenger RNA strand)

The sense and antisense oligonucleotides to be synthesized in this case are the following (order the smallest scale oligonucleotide synthesis available; no extra purification is necessary):

Sense oligo: 5' AAA**G-N_2-N_3-N_4-…-N_{18}-N_{19}** 3' (22 bases);

Antisense oligo: 5' AAAA**N'$_{19}$-N'$_{18}$-…-N'$_4$-N'$_3$-N'$_2$** 3' (22 bases).

Here nucleotide **N'$_{19}$** is a complementary counterpart to **N_{19}**, **N'$_{18}$** – to **N_{18}** and so on. Uncomplementary overhangs AAAG and AAAA serve as cohesive ends for effective and orientation-defined cloning of synthetic DNA fragment into linearized vectors.

Example: an efficient target sequence for human p53 tumor suppressor gene is GACTCCAGTGGTAATCTAC (8). Corresponding sense and antisense oligonucleotides are:

AAAGACTCCAGTGGTAATCTAC (si-p53 Sense Oligo),

AAAAGTAGATTACCACTGGAGT (si-p53 Antisense Oligo).

If the target sequence starts from the nucleotide N_1 other than G, the sense oligonucleotide should have additional G base:

Sense oligo: 5' AAAG-**N_1-N_2-N_3-N_4-…-N_{18}-N_{19}** 3' (23 bases);

Antisense oligo: 5' AAAA**N'$_{19}$-N'$_{18}$-…-N'$_4$-N'$_3$-N'$_2$-N'$_1$** 3' (23 bases).

If the target sequence is more than 19 bases long, the oligonucleotides should be correspondingly extended. In any case the sense oligo should start from AAAG, and the antisense oligo from AAAA.

3. Cloning into psiRNA, psiTEST-target-LUC, psiTEST-LUC-target

1. Dilute the aliquotes of sense and antisense oligonucleotides by TE buffer (10 mM Tris-HCl, pH 7.5, 0.2 mM EDTA) to obtain a concentration of 1÷2 µg/ml (equivalent to 0.1÷ 0.3 µM or A_{260} = 0.03÷0.07).

2. Set up 20 µl ligation reactions, three reactions per each target sequence (si-p53 Sense and Antisense oligonucleotides are given in the kit to make positive control). Combine the following reagents in three separate Eppendorf tubes, one for each linearized vector:

14 µl	TE buffer;
1 µl	Linearized Vector (psiRNA, or psiTEST-target-LUC, or psiTEST-LUC-target);
1 µl	Diluted Sense Oligonucleotide;
1 µl	Diluted Antisense Oligonucleotide;
2 µl	Cloning Enzyme 10X Reaction Buffer;
1 µl	Cloning Enzyme Mix.

   Incubate the ligation mixtures 2 h at 37°C or overnight at room temperature.

3. Transform competent *E.coli* cells (XL1-Blue or DH5α strain) with the ligation products.

4. Plate the transformed *E.coli* cells on LB plates containing 100 – 200 µg/ml Ampicillin and grow overnight at 37°C.

5. Pick clones (typically one clone per ligation reaction is enough, there is no need to prepare minipreps), grow liquid cultures in 5 – 50 ml of LB medium containing 100 – 200 µg/ml Ampicillin and isolate plasmid DNA. To ensure optimal purity of plasmid DNA for efficient transfection, use glass-fiber filter binding column technique (we routinely use QIAGEN Plasmid Midi Kit).

6. Adjust final concentration of each purified plasmid to 0.5 mg/ml with TE buffer.

4. Plasmid Verification

Confirm the presence of inserted fragment in recombinant plasmids by PCR or DNA sequencing.

For 50 µl amplification reaction take 0.5 µg of recombinant plasmid as a template, 1 µl of corresponding Sequence/PCR Forward Primer, and 1 µl of the same Antisense Oligonucleotide which was used for plasmid generation as PCR reverse primer (adjust Antisense Oligonucleotide concentration to 10 µM). The PCR conditions are flexible, we use the following protocol:

95°C, 30 sec – denaturation;
60°C, 1 min – annealing;
70°C, 1 min – extention.

Typically, 20 rounds are enough to see the product of the following size:

195 bp for psiRNA vector derivatives;
189 bp for psiTEST-target-LUC vector derivatives;
198 bp for psiTEST-LUC-target vector derivatives.

For DNA sequencing use the corresponding Sequence/PCR Forward Primer. Refer to Yorkshire Bioscience Web-site http://www.york-bio.com for vector sequences. psiRNA, psiTEST-target-LUC, and psiTEST-LUC-target functional maps are shown in the Section V.

5. Planning of Transfection Experiments

Once a panel of target sequences T1, T2, … , Tn had been cloned into psiRNA, psiTEST-target-LUC, and psiTEST-LUC-target vectors, we suggest the following experimental plan for testing of target eficiencies in transient transfection assay:

Plasmid Combination (2:1)		**Comments**
Effector Plasmid	**Reporter Plasmid**	
psiRNA-T1	**psiTEST-T1-LUC**	Testing the RNA interference effect when the target is placed 5' to Luciferase coding region
psiRNA-negative	**psiTEST-T1-LUC**	
psiRNA-T1	**psiTEST-LUC-T1**	Testing the RNA interference effect when the target is placed 3' to Luciferase coding region
psiRNA-negative	**psiTEST-LUC-T1**	
psiRNA-T1	**psiTEST-LUC-control** **(or psiTEST-control-LUC)**	Testing the off-target influence of the effector plasmid to Luciferase gene expression (due to effector plasmid impurities and/or occasional T1 siRNA homology to Luciferase coding sequence)
psiRNA-negative	**psiTEST-LUC-control** **(or psiTEST-control-LUC)**	
…	**…**	**…**
psiRNA-Tn	**psiTEST-Tn-LUC**	**…**
psiRNA-negative	**psiTEST-Tn-LUC**	
psiRNA-Tn	**psiTEST-LUC-Tn**	
psiRNA-negative	**psiTEST-LUC-Tn**	
psiRNA-Tn	**psiTEST-LUC-control** **(or psiTEST-control-LUC)**	
psiRNA-negative	psiTEST-LUC-control (or psiTEST-control-LUC)	In fact, it is not necessary to repeat this combination for every target sequence
psiRNA-siLUC	**psiTEST-LUC-control** **(or psiTEST-control-LUC)**	To have a positive control for RNA interference effect

A sequence GTCACACAATCAGCGGAGA from rat Heat Shock Protein HSP-27 cDNA with poor RNA interference ability had been cloned in psiRNA-negative vector to generate control effector plasmid with no RNA interference effect.

A sequence GATTCAGGTCAAAGACAGC from human Transferrin Receptor cDNA with poor RNA interference ability is used as target sequence in psiTEST-LUC-control and psiTEST-control-LUC reporter plasmids. No siRNA is produced by these two plasmids, thus the endogenous transferrin receptor expression may not be affected.

A sequence GTGCGTTGCTAGTACCAAC from *Photinus pyralis* Luciferase cDNA had been cloned in psiRNA-siLUC plasmid to generate positive control effector plasmid with strong RNA interference effect against firefly Luciferase.

6. Cell Transfection

In general, any mammalian cells exhibiting high level of tranfection are suitable for siRNA target testing. We routinely use HEK-293 (Human Embryonic Kidney) cell line in 24-well plate format, although other tissue culture formats could be used as well. For better accuracy we recommend to use 2 to 4 wells per each combination of effector / reporter plasmids. The optimal proportion between effector and reporter plasmids is 2:1.

1. Calculate how many wells will be used for cell transfection experiment according to the plan presented in the Paragraph 5 of this Section.

2. Seed approximately 50 000 cells into each well of 24-well plate. If using other well format, calculate required cell number per well according to well surface. To produce comparable data, it is crucially important to seed equal amount of cells into each well. We routinely prepare large volume of cell suspension with concentration about 50 000 cells per 1 ml of growth medium and then transfer this suspension, 1 ml per well, to all wells. For homogeneous cell distribution the suspension should be permanently pipetted during the whole process of seeding.

3. Next day after seeding transfect the cells with various plasmid combinations according to the plan. The choice of transfection procedure depends on the cell type being used. For HEK-293 cells we routinely use lipofection technique with Lipofectamin-2000, INVITROGEN. It is important to prepare all mixtures with high accuracy, especially when handling small volumes of DNA or transfection compound.

4. Measure Luciferase expression level 48 hours after transfection.

7. Luciferase Measurements

Any conventional technique could be used for Luciferase expression level determination. We employ Luciferase Reporter Assay Kit from BD Biosciences.

48 h after transfection remove growth medium and prepare cellular lysate from each well of transfected cells in fixed volume of lysis buffer. Follow Luciferase assay protocol. It is not necessary to normalize cellular lysates to the protein content if aliquotes of equal volumes are used for Luciferase activity measurements.

8. Analysis of Results

If values A, B, C, D, E, F, and G, expressed in Luciferase Relative Light Units, are obtained for each effector/reporter plasmid combination as shown in the table below, calculate 5'- and 3'- Relative Luciferase Activity (5'-RLA and 3'-RLA) using the following formulas:

$$\textbf{5'-RLA (T1)} = \frac{(A/B)}{(E/F)} \cdot 100\% \;; \qquad \textbf{3'-RLA (T1)} = \frac{(C/D)}{(E/F)} \cdot 100\% \;.$$

Plasmid Combination (2:1)		Results
Effector Plasmid	**Reporter Plasmid**	
psiRNA-T1	**psiTEST-T1-LUC**	**A**
psiRNA-negative	**psiTEST-T1-LUC**	**B**
psiRNA-T1	**psiTEST-LUC-T1**	**C**
psiRNA-negative	**psiTEST-LUC-T1**	**D**
psiRNA-T1	**psiTEST-LUC-control (or psiTEST-control-LUC)**	**E**
psiRNA-negative	**psiTEST-LUC-control (or psiTEST-control-LUC)**	**F**
psiRNA-siLUC	**psiTEST-LUC-control (or psiTEST-control-LUC)**	**G**

Dividing by (E/F) allows for eliminating the off-target influence of the effector plasmid psiRNA-T1 to Luciferase gene expression (due to effector plasmid impurities and/or occasional T1 siRNA homology to Luciferase coding sequence). If the same plasmid combination is tested in duplicates or triplicates, use corresponding average values in the above-mentioned formulas.

Please note that for different reporter plasmids the Luciferase expression level may vary substantially, either due to reporter plasmid impurities or to the interference of the target sequence with ribosomal binding and translation initiation in case of psiTEST-target-LUC derivatives (where the target sequence is located immediately upstream of the Luciferase translation start codon). Such kind of variations should not affect the target efficiency estimation because for each target sequence the influence

of siRNA-producing effector plasmid is compared to the negative control effector plasmid on the same reporter plasmid.

Calculate also the Mean Relative Luciferase Activity (M-RLA) for each target sequence using the following formula:

$$\textbf{M-RLA (T1)} = \frac{1}{2}\textbf{(5'-RLA (T1) + 3'-RLA (T1))} \ .$$

The effective control target against human p53 tumor suppressor gene, made of si-p53 Sense and Antisense Oligos (provided in the kit), gives usually the M-RLA value close to 10%. The effector plasmid psiRNA-siLUC used for positive control gives typically the Control Relative Luciferase Activity value (C-RLA calculated as G/F) about 5%. We propose the following range of siRNA target sequences depending on the M-RLA obtained:

M-RLA	Target Sequence
less than 10%	Excellent
10% – 20%	Good
20% – 30%	Acceptable
more than 30%	Bad

V. Vector Backbones, Control targets, and Oligonucleotide Sequences

1. psiRNA vector

Location of features (Fig. 4).

Human U6 small nuclear RNA promoter:	7 – 264;
Sequence/PCR forward primer:	93 – 118;
siRNA cloning site:	262 – 297;
Human H1 small nuclear RNA promoter:	393 – 294;
pUC plasmid replication origin:	1476 – 833;
Site of replication initiation:	864;
Ampicillin resistance gene (β-lactamase):	2484 – 1627;
SV40 early mRNA polyadenylation signal:	3517 – 3271;
Puromycin resistance gene:	4187 – 3591;
Human phosphoglycerate kinase (PGK) promoter:	4711 – 4208.

Complete psiRNA vector sequence is available at: www.york-bio.com/

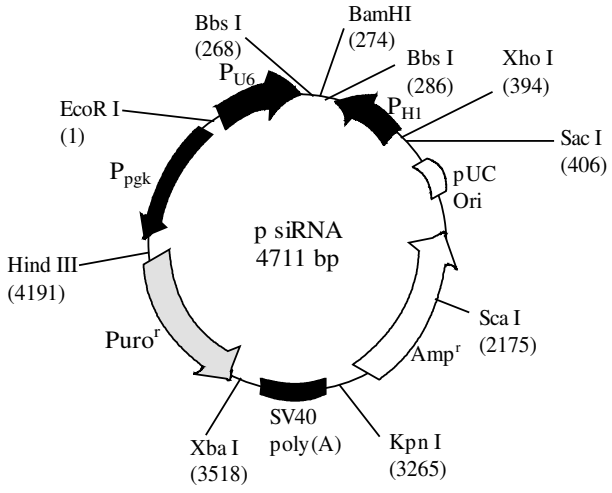

Fig. 4. psiRNA vector map.

2. psiTEST-target-LUC vector

Location of features (Fig. 5).

Human cytomegalovirus (CMV) immediate early promoter:	209 – 863;
Sequence/PCR forward primer:	738 – 759;
Target cloning site:	901 – 936;
Firefly Luciferase coding region:	1016 – 2665;
Bovine growth hormone (BGH) mRNA polyadenylation signal:	2718 – 2948;
pUC plasmid replication origin:	4108 – 3465;
Site of replication initiation:	3496;
Ampicillin resistance gene (β-lactamase):	5116 – 4259

Complete psiTEST-target-LUC vector sequence is available at: www.york-bio.com/

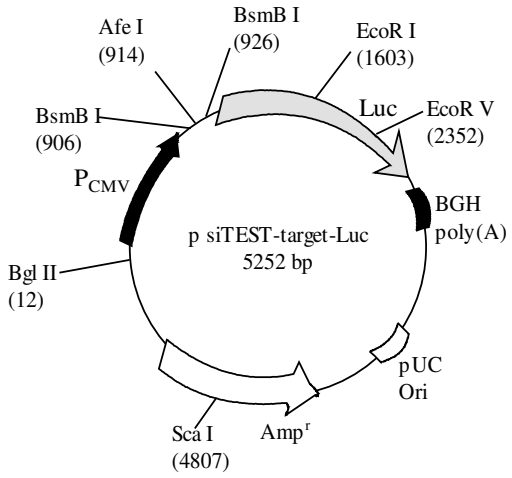

Fig. 5. psiTEST-target-LUC vector map.

3. psiTEST-LUC-target vector

Location of features (Fig. 6).

Human cytomegalovirus (CMV) immediate early promoter:	209 – 863;
Firefly Luciferase coding region:	919 – 2568;
Sequence/PCR forward primer:	2466 – 2487;
Target cloning site:	2638 – 2673;
Bovine growth hormone (BGH) mRNA polyadenylation signal:	2701 – 2931;
pUC plasmid replication origin:	4091 – 3448;
Site of replication initiation:	3479;
Ampicillin resistance gene (β-lactamase):	5099 – 4242.

Complete psiTEST-LUC-target vector sequence is available at:
www.york-bio.com/

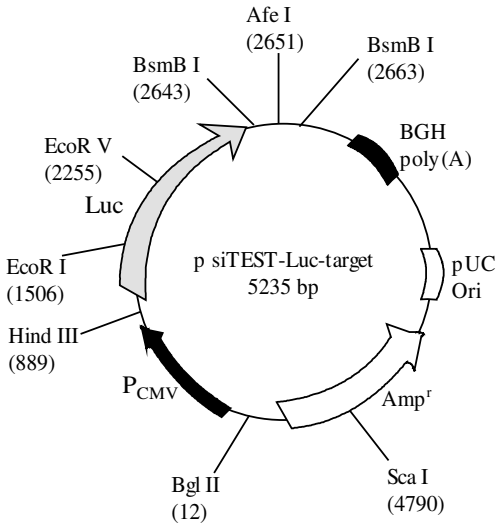

Fig. 6. psiTEST-LUC-target vector map

4. Oligonucleotide sequences

si-p53 Sense Oligo:	AAAGACTCCAGTGGTAATCTAC ;
si-p53 Antisense Oligo:	AAAAGTAGATTACCACTGGAGT ;
Sequence/PCR Forward Primer for psiRNA:	GACTGTAAACACAAAGATATTAGTAC ;
Sequence/PCR Forward Primer for psiTEST-target-LUC:	CCAAAATGTCGTAACAACTCCG ;
Sequence/PCR Forward Primer for psiTEST-LUC-target:	TGTGTTTGTGGACGAAGTACCG .

5. Control target sequences

psiRNA-negative target sequence:	GTCACACAATCAGCGGAGA ;
psiRNA-siLUC target sequence:	GTGCGTTGCTAGTACCAAC ;
psiTEST-control-LUC and psiTEST-LUC-control target sequence:	GATTCAGGTCAAAGACAGC .

6. Patents/Disclaimers

CMV-promoter: U.S. Patent No. 5,168,062 and No. 5,385,839 assigned to the University of Iowa Research Foundation.

BGH poly(A): U.S. Patent No. 5,122,458, Europian Patent No. 0 173 552 and Japanese Patent No. 1955752 owned patent rights by Research Corporation Technologies (RCT).

VI. References

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