Luciferase-based ENGENIS™ siRNA Test System (25x)
Catalog # N0070

INSTRUCTION MANUAL

I. General Purpose

Luciferase-based ENGENIS™ (Entire **Gene**/cDNA or **I**solated **S**equence**) siRNA Test System** is designed to provide a quantitative approach for the evaluation of the potential of any DNA sequence to serve as an efficient target for RNA interference (RNAi). Current computer-based RNAi target searching algorithms are not perfect, leaving the probability of a selected sequence to be a good target from 30% to 60%. It is therefore clear that the ability of a computer-selected sequence to induce RNA interference has to be confirmed experimentally. The **ENGENIS™ siRNA Test System** allows the screening of 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. The distance between the target site and reporter gene coding region along the mutual messenger RNA also 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. This kind of effects may obscure the real efficiency of particular targets.

Unlike other kits, the **ENGENIS™ siRNA Test System** offers two possibilities for the evaluation of the target sequence efficiency: using entire gene technology and using technology with isolated target sequences *per se*. In the latter case, short target sequences are located at defined positions immediately upstream or downstream of the Luciferase gene coding region. For the entire gene technology, two variants of transcript can be generated by placing the gene of interest either in front of Luciferase coding region or behind it.

Independent evaluation of the reporter gene silencing corresponding to two different positions of isolated target sequence or entire target gene permits to avoid or reduce the influence of the target accessibility factor and other factors related to RNA conformation, thus making comparison between various targets more reliable. Another advantage of the **ENGENIS™ siRNA Test System** is that it makes possible the 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 **ENGENIS™ siRNA Test System** consists mainly of four vectors: one effector plasmid (psiRNA) producing double-stranded siRNA, and three reporter plasmids (psiTEST-target-LUC, psiTEST-LUC-target, psiTEST-target-IRES-LUC) expressing Luciferase reporter gene fused to siRNA target sequence or the target gene cDNA.

siRNA testing on isolated targets (Fig. 1) 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 three linearized plasmids to generate siRNA coding sequence in effector plasmid psiRNA and siRNA target sequence in two reporter plasmids psiTEST-target-LUC and psiTEST-LUC-target.

Within the framework of the entire gene technology, the **ENGENIS™ siRNA Test System** provides two supercoiled vectors psiTEST-LUC-target and psiTEST-target-IRES-LUC containing multiple cloning sites (MCS) intended for target gene cloning and subsequent generation of a fused transcript (Fig. 2). Encephalomyocarditis virus Internal Ribosomal Binding Site (IRES) of the reporter plasmid psiTEST-target-IRES-LUC enables Luciferase translation from the second cistron of the fused transcript. MCS structures and vector backbones are described in Section VI.

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 allows us 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. 3. 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, the 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. The 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.

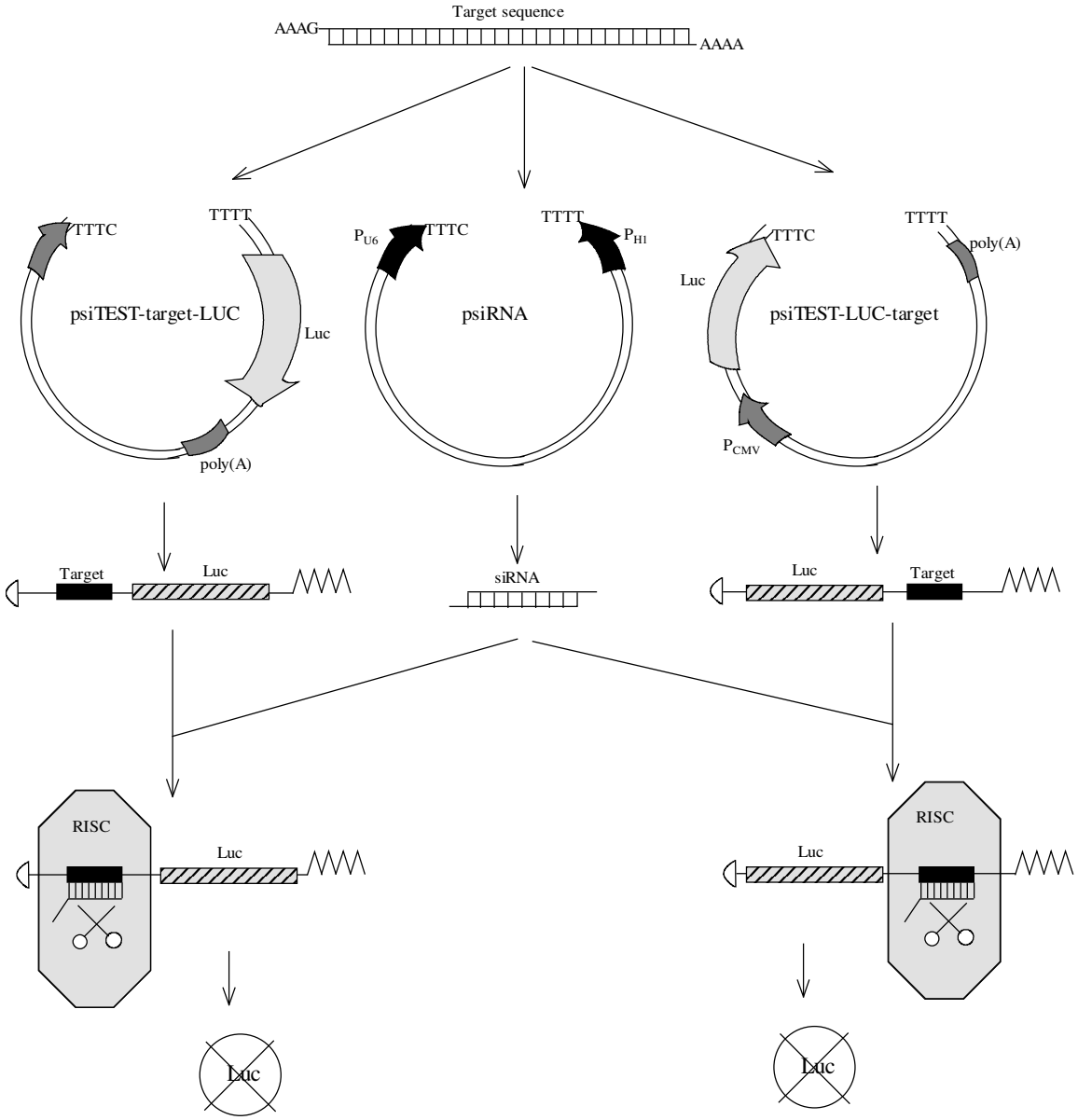


Fig. 1. Construction and operation of effector and reporter plasmids for siRNA testing on isolated targets.

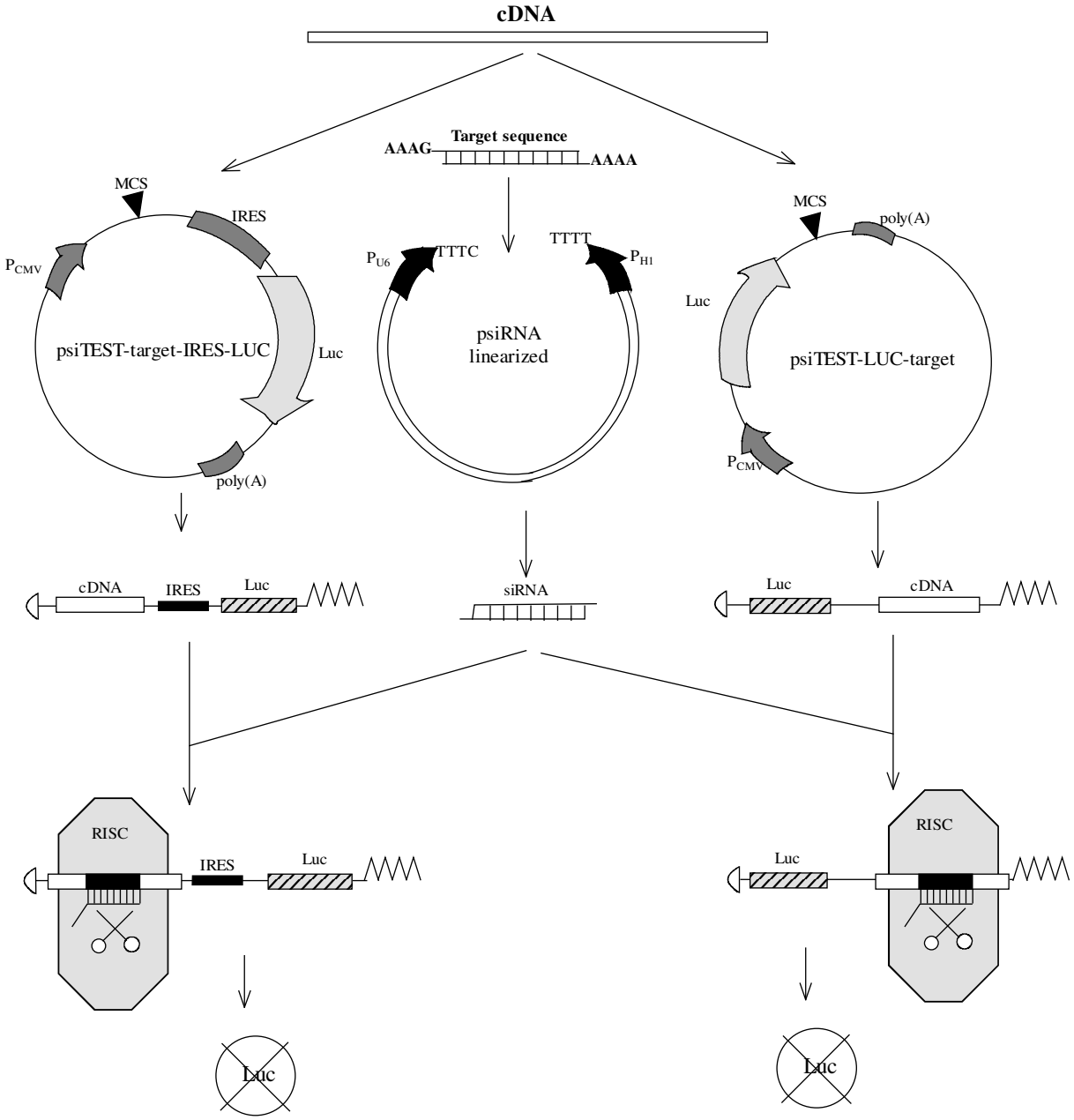


Fig. 2. Construction and operation of effector and reporter plasmids for siRNA target testing on fused mRNA.

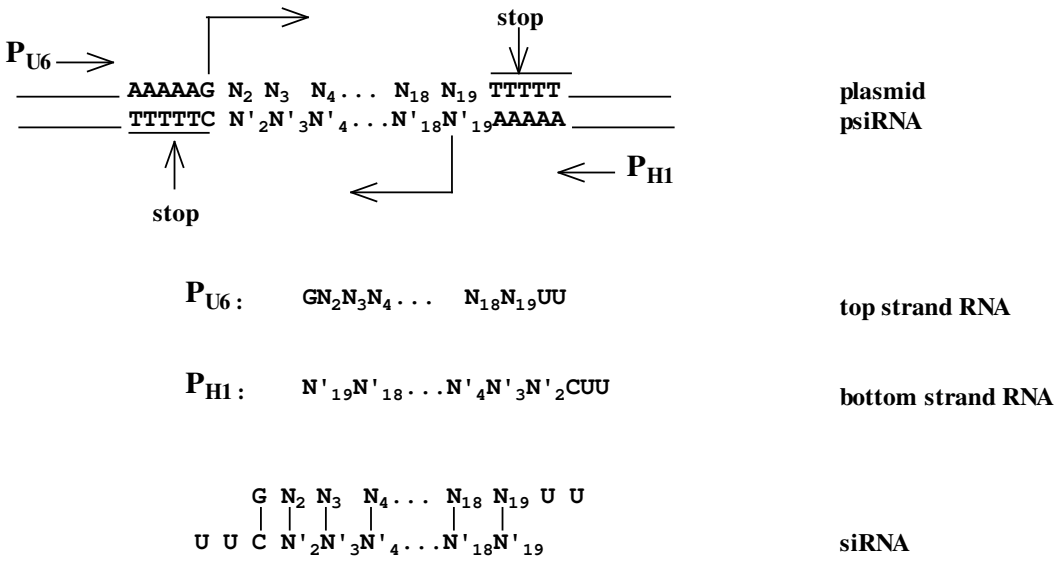
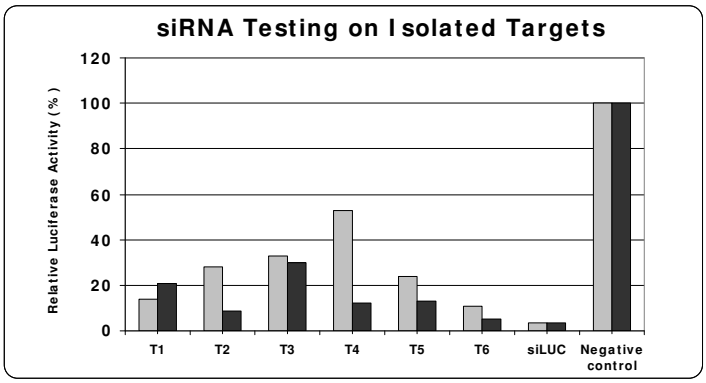


Fig. 3. siRNA expression from the dual promoter effector plasmid psiRNA.

2. Model experiments

To demonstrate the utility of **ENGENIS™ siRNA Test System**, 6 target sequences against Human Transferrin receptor (CD71) were chosen for the screening of their gene silencing potential. Corresponding oligonucleotides were synthesized and sub-cloned into linearized effector (psiRNA) and reporter plasmids (psiTEST-target-LUC and psiTEST-LUC-target). In parallel, CD71 cDNA was sub-cloned into psiTEST-LUC-target and psiTEST-target-IRES-LUC reporter 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. 4).

A



B

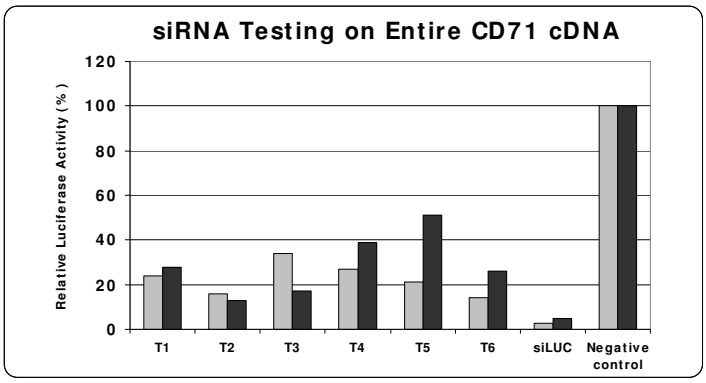


Fig. 4. Target efficiency determination by **ENGENIS™ siRNA Test System**. 6 target sequences (T1—T6) from CD71 gene were tested. Effector plasmid psiRNA-siLUC expressing siRNA against Luciferase was used as a positive control, and effector plasmid psiRNA-negative expressing irrelevant siRNA was used as a negative control. Cells HEK-293 were cotransfected with mixtures (2:1) of siRNA-producing effector plasmid and corresponding reporter plasmid bearing isolated target (A) or entire CD71 cDNA (B). Luciferase activity was measured 48 h post transfection, and relative Luciferase activity was calculated for each reporter construct derived from psiTEST-target-LUC or psiTEST-target-IRES-LUC (light grey columns, target site is located upstream of the Luciferase coding region) and psiTEST-LUC-target (dark grey columns, target site is located downstream of the Luciferase coding region).

Both technologies were employed to measure Luciferase gene silencing: isolated sequence technology (Fig. 4A) and entire gene technology (Fig. 4B). As a positive control, an effector plasmid psiRNA-siLUC expressing highly efficient siRNA against *Photinus pyralis* Luciferase was cotransfected with each type of the reporter constructs. As a negative control, effector plasmid psiRNA-negative expressing irrelevant siRNA was used to give Luciferase activity reference point (100%).

As shown in Fig. 4, three out of six targets (T1, T2, and T6) demonstrate significant silencing potential (relative Luciferase activity less then 30%) when placed both upstream and downstream of the Luciferase coding region.

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-target-LUC	supercoiled	(500 ng/µl)	25 µl
7	psiTEST-LUC-target	supercoiled	(500 ng/µl)	25 µl
8	psiTEST-target-IRES-LUC	supercoiled	(500 ng/µl)	25 µl
9	si-p53 Sense Oligo		(10 µM)	25 µl
10	si-p53 Antisense Oligo		(10 µM)	25 µl
11	Sequence/PCR Forward Primer for psiRNA		(10 µM)	50 µl
12	Sequence/PCR Forward Primer for psiTEST-target-LUC and psiTEST-target-IRES-LUC		(10 µM)	50 µl
13	Sequence/PCR Forward Primer for psiTEST-LUC-target		(10 µM)	50 µl
14	Cloning Enzymes Mix			100 µl
15	Cloning Enzymes 10X Reaction Buffer			200 µl
16	Manual			

IV. siRNA testing on isolated targets

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 free internet access, which gave consistent results in our experience:

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 **ENGENIS™ siRNA Test System** 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 **ENGENIS™ siRNA Test System** 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 a product of the following size:

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

5. Planning of Transfection Experiments

Once a panel of target sequences T1, T2, … , Tn has 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-target** **(or psiTEST-target-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-target** **(or psiTEST-target-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-target** **(or psiTEST-target-LUC)**	
psiRNA-negative	psiTEST-LUC-target (or psiTEST-target-LUC)	In fact, it is not necessary to repeat this combination for every target sequence
psiRNA-siLUC	**psiTEST-LUC-target** **(or psiTEST-target-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 has been cloned in psiRNA-negative vector to generate control effector plasmid with no RNA interference effect.

A sequence GTGCGTTGCTAGTACCAAC from *Photinus pyralis* Luciferase cDNA has 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 greater accuracy we recommend to use 2 to 4 wells per each combination of effector / reporter plasmids. The optimal ratio of effector plasmids to reporter plasmids is 2:1.

1. Calculate the number of wells to be used for cell transfection experiment according to the plan presented in 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 vitally important to seed equal amount of cells into each well. We routinely prepare large volumes of cell suspension with concentration of 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. The 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 great 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 can 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-target** (or psiTEST-target-LUC)	**E**
psiRNA-negative	**psiTEST-LUC-target** (or psiTEST-target-LUC)	**F**
psiRNA-siLUC	**psiTEST-LUC-target** (or psiTEST-target-LUC)	**G**

Dividing by (E/F) allows to eliminate 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 the case of psiTEST-target-LUC derivatives (where the target sequence is located immediately upstream of the Luciferase translation start codon). This kind of variations should not

affect the target efficiency estimation as the influence of siRNA-producing effector plasmid for each target sequence 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 **ENGENIS™ siRNA Test System**), usually gives an M-RLA value close to 10%. The effector plasmid psiRNA-siLUC used for positive control typically gives a Control Relative Luciferase Activity value (C-RLA calculated as G/F) of about 5%. We suggest the following ratings 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. siRNA Target Testing on Fused mRNA

1. Selection of Target Sequences and Design of Oligonucleotides

For target sequence selection see Paragraph 1 of Section IV. For oligonucleotides design follow instructions in Paragraph 2 of Section IV.

2. Cloning of Oligonucleotides into psiRNA Effector Plasmid

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, one reaction per each target (si-p53 Sense and Antisense oligonucleotides are given in the **ENGENIS™ siRNA Test System** to make positive control). Combine the following reagents in the Eppendorf tubes:

14 µl	TE buffer;
1 µl	Linearized psiRNA Vector;
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 legation 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.

3. Cloning of Target Gene cDNA into psiTEST-LUC-target and psiTEST-target-IRES-LUC Reporter Plasmids

Use multiple cloning sites of psiTEST-LUC-target and psiTEST-target-IRES-LUC (see Section VI for MCS and vector backbone details) to sub-clone the cDNA corresponding to the gene of interest. Cloning Enzyme Mix and Cloning Enzyme 10X Reaction Buffer can be used for ligation procedure as described in Paragraph 2 of this Section. If no full-size cDNA is available, cDNA PCR fragments of the gene of interest containing several target sites may be employed for cloning as well.

Grow clones in minipreps, select correct constructs by restriction enzyme analysis, then grow and purify selected plasmids. To ensure optimal purity of plasmid DNA for efficient transfection, use glass-fiber filter binding column technique (we routinely use QIAGEN Plasmid Midi Kit).

4. Plasmid Verification

Confirm the presence of inserted oligonucleotide fragment in recombinant effector plasmids by PCR or DNA sequencing. For 50 µl amplification reaction take 0.5 µg of generated effector plasmid as a template, 1 µl of Sequence/PCR Forward Primer for psiRNA, 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 a product of 195 bp.

For DNA sequencing of psiRNA-derivatives use the Sequence/PCR Forward Primer for psiRNA. Refer to Yorkshire Bioscience Web-site http://www.york-bio.com for psiRNA vector sequence.

Confirm the presence of target cDNA fragment in recombinant reporter plasmids by restriction analysis or DNA sequencing. psiTEST-target-IRES-LUC and psiTEST-LUC-target functional maps are shown in Section VI.

For DNA sequencing use the corresponding Sequence/PCR Forward Primers. Refer to Yorkshire Bioscience Web-site for psiTEST-target-IRES-LUC and psiTEST-LUC-target vector sequences.

5. Planning of Transfection Experiments

Once reporter plasmids psiTEST-IRES-cDNA-LUC and psiTEST-LUC-cDNA bearing target gene cDNA have been generated and a panel of target sequences T1, T2, … , Tn has been cloned into effector plasmid psiRNA, we suggest the following experimental plan for the testing of target efficiencies in transient transfection assay:

Plasmid Combination (2:1)		**Comments**
Effector Plasmid	**Reporter Plasmid**	
psiRNA-T1	**psiTEST-cDNA-IRES-LUC**	Testing the RNA interference effect when the target gene cDNA is placed 5' to Luciferase coding region
psiRNA-T2	**psiTEST-cDNA-IRES-LUC**	
…	**…**	
psiRNA-Tn	**psiTEST-cDNA-IRES-LUC**	
psiRNA-negative	**psiTEST-cDNA-IRES-LUC**	To have a negative control for RNA interference effect
psiRNA-siLUC	**psiTEST-cDNA-IRES-LUC**	To have a positive control for RNA interference effect
psiRNA-T1	**psiTEST-LUC-cDNA**	Testing the RNA interference effect when the target gene cDNA is placed 3' to Luciferase coding region
psiRNA-T2	**psiTEST-LUC-cDNA**	
…	**…**	
psiRNA-Tn	**psiTEST-LUC-cDNA**	
psiRNA-negative	**psiTEST-LUC-cDNA**	To have a negative control for RNA interference effect
psiRNA-siLUC	**psiTEST-LUC-cDNA**	To have a positive control for RNA interference effect
psiRNA-T1	**psiTEST-LUC-target**	Testing the off-target influence of the effector plasmids to Luciferase gene expression (due to effector plasmid impurities and/or occasional T1, T2, …, Tn siRNA homology to Luciferase coding sequence)
psiRNA-T2	**psiTEST-LUC-target**	
…	**…**	
psiRNA-Tn	**psiTEST-LUC-target**	
psiRNA-negative	**psiTEST-LUC-target**	
psiRNA-siLUC	psiTEST-LUC-target	Optional

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

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

6. Cell Transfection and Luciferase Measurements

For cell transfection follow instructions in Paragraph 6 of Section IV.

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

For Luciferase expression level determination see Paragraph 7 of Section IV.

7. Analysis of Results

If values A, B, C, D, E, F, G, and H, 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:

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

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

Dividing by (G/H) allows to eliminate 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 reporter plasmids derived from psiTEST-target-IRES-LUC display about 10 times lower level of Luciferase expression than the plasmids derived from psiTEST-LUC-target. This is due to a low efficiency of ribosomal binding to the Internal Ribosomal Entry Site (IRES).

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

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

The effector plasmid psiRNA-siLUC used for positive control typically gives a Control Relative Luciferase Activity value (C-RLA calculated as $\frac{1}{2}$(C/B + F/E)) of about 5%.

We suggest the following rating of siRNA target sequences depending on the M-RLA obtained:

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

VI. Vector Backbones, Control targets, and Oligonucleotide Sequences

1. psiRNA vector

Location of features (Fig. 5).

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.

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

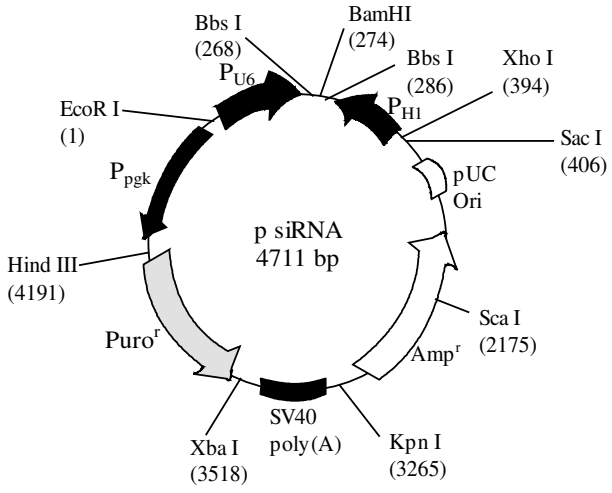


Fig. 5. psiRNA vector map.

2. psiTEST-target-LUC vector

Location of features (Fig. 6).

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

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

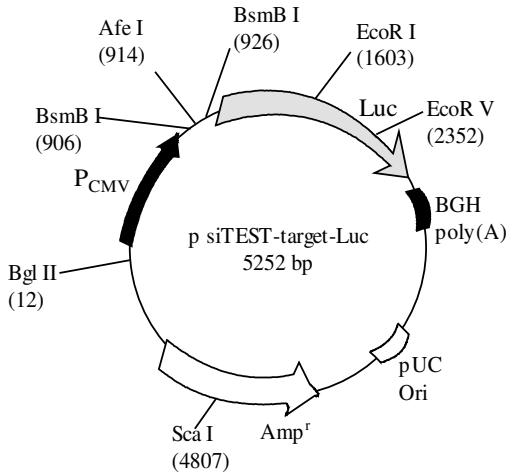


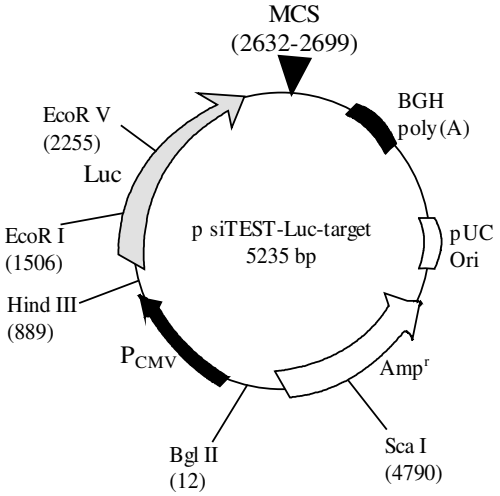
Fig. 6. psiTEST-target-LUC vector map.

3. psiTEST-LUC-target vector

Location of features (Fig. 7).

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;
Multiple Cloning Site:	2632 – 2699;
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.

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



BsmB I Afe I BsmB I Xho I BamH I
GGTACCAAA GCGAGACGCT AGCGCTCCCG GGCGTCTCAT TTTGCGGCCG CCTCGAGGGG CCCGGATCC
Kpn I Nhe I Sma I Not I Apa I

Fig. 7. psiTEST-LUC-target vector map and Multiple Cloning Site

4. psiTEST-target-IRES-LUC vector

Location of features (Fig. 8).

Human cytomegalovirus (CMV) immediate early promoter:	209 – 863;
Sequence/PCR forward primer:	738 – 759;
Multiple Cloning Site:	899 – 933;
Encephalomyocarditis virus Internal Ribosomal Entry Site (IRES):	941 – 1526;
Firefly Luciferase coding region:	1529 – 3178;
Bovine growth hormone (BGH) mRNA polyadenylation signal:	3271 – 3461;
pUC plasmid replication origin:	4621 – 3978;
Site of replication initiation:	4009;
Ampicillin resistance gene (β-lactamase):	5629 – 4772.

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

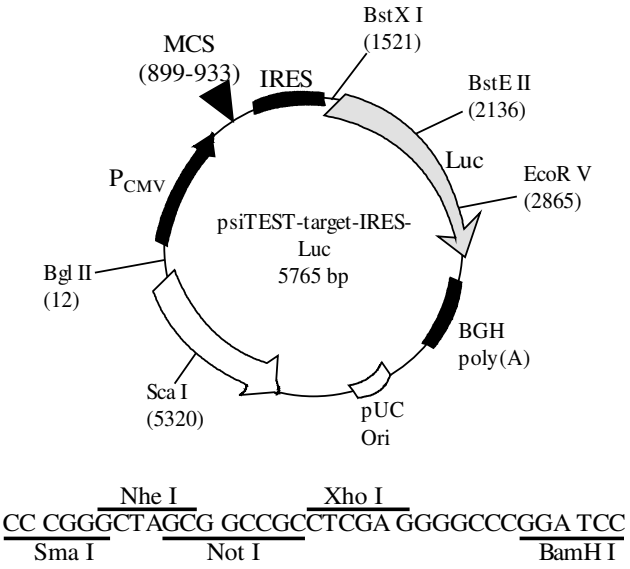


Fig. 8. psiTEST-target-IRES-LUC vector map and Multiple Cloning Site

5. 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 and psiTEST-target-IRES-LUC: CCAAAATGTCGTAACAACTCCG ;

Sequence/PCR Forward Primer for psiTEST-LUC-target: TGTGTTTGTGGACGAAGTACCG .

6. Control target sequences

psiRNA-negative target sequence: GTCACACAATCAGCGGAGA ;

psiRNA-siLUC target sequence: GTGCGTTGCTAGTACCAAC .

7. 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, European Patent No. 0 173 552 and Japanese Patent No. 1955752 owned patent rights by Research Corporation Technologies (RCT).

VII. References

1. Zheng L., Liu J., Batalov S., Zhou D., Orth A., Ding S., and Schultz P.G. (2004) An approach to genomewide screens of expressed small interfering RNAs in mammalian cells. *Proc. Natl. Acad. Sci. USA*, **101**, 135-140.
2. Kaykas A. and Moon R.T. (2004) A plasmid-based system for expressing small interfering RNA libraries in mammalian cells. *BMC Cell Biol*., **5**, 16.
3. Martinez J., Patkaniowska A., Urlaub H., Luhrmann R., and Tuschl T. (2002) Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. *Cell*, **110**, 563-574.
4. Reynolds A., Leake D., Boese Q., Scarine S., Marshall W.S., and Khvorova A. (2004) Rational siRNA design for RNA interference. *Nat. Biotechnol.*, **22**, 326-330.
5. Ui-Tei K., Naito Y., Takahashi F., Haraguchi T., Ohki-Hamazaki H., Juni A., Ueda R., and Saigo K. (2004) Guidelines for the selection of highly effective siRNA sequences for mammalian and chick RNA interference. *Nucleic Acids Res*., **32**, 936-948.
6. Bridge A.J., Pebernard S., Ducraux A., Nicoulaz A.L., and Iggo R. (2003) Induction of an interferon response by RNAi vectors in mammalian cells. *Nat Genet*., **34**, 263-264.
7. Fish R.J. and Kruithof E.K. (2004) Short-term cytotoxic effects and long-term instability of RNAi delivered using lentiviral vectors. *BMC Mol. Biol*., **5**, 9.
8. Brummelkamp T.R., Bernards R., and Agami R. (2002) A system for stable expression of short interfering RNAs in mammalian cells. *Science*, **296**, 550-553.