MICRORNA BIOSENSORS: APPLICATION FOR THE PSICHECK™-2 VECTOR

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MicroRNA regulation of gene expression is the focus of intense research. Tools for simple, rapid screening of miRNA activity will be essential to progress in this field. Here we demonstrate the utility of the psiCHECK™-2 Vector, originally designed for screening siRNA silencing efficiency, as a biosensor for microRNA activity in cells.

Introduction

MicroRNAs (miRNAs) are short, noncoding RNAs that regulate gene expression. miRNAs target the 3´-untranslated region of messenger RNA sequences, affect RNA stability and prevent translation (1). More than 200 miRNAs have been identified in humans, and expression profiling studies have implicated them in a wide range of biological processes and disease states including development, metabolism, and cancer. Efforts are now focused on understanding miRNA function and mechanisms of regulation. These efforts require simple and rapid assays for monitoring miRNA activity and screening for potential miRNA targets.

The psiCHECK™ Vectors[a–f] provide a quantitative and rapid approach for initial optimization of RNA interference (2). These vectors also are ideal for examining the effect of 3´ untranslated regions (3´ UTRs), such as miRNA target sequences, on gene expression. psiCHECK™ Vectors contain a multiple cloning region downstream of the stop codon of an SV40 promoter-driven *Renilla* luciferase gene (Figure 1). This allows expression of a *Renilla* transcript with the 3´ UTR sequence of interest. *Renilla* luciferase activity is then used to assess the effect of the 3´ UTR on transcript stability and translation efficiency.

The psiCHECK™-2 Vector, unlike most other luciferase reporter vectors, also contains a constitutively expressed firefly luciferase gene. Firefly luciferase is used to normalize transfections and eliminates the need to transfect a second vector control. Because miRNAs affect translation and not transcriptional activation, both the miRNA reporter and internal control can be expressed from the same plasmid without the danger of the miRNA reporter regulation affecting the control. Here we demonstrate the use of the psiCHECK™-2 Vector as a biosensor for miRNA-21 (miR-21) in different cell lines and as a tool to test an miRNA target.

Generating an miRNA Biosensor

To test the utility of psiCHECK™-2 Vector as a biosensor for miRNA activity, we selected miR-21 as a model. The relative levels of miR-21 have been determined for different cell lines and tissues through a variety of techniques including Northern blotting and bead-based expression profiling (3,4; Table 1). Relative differences between HEK293 and HeLa cells were confirmed by real-time PCR (data not shown).

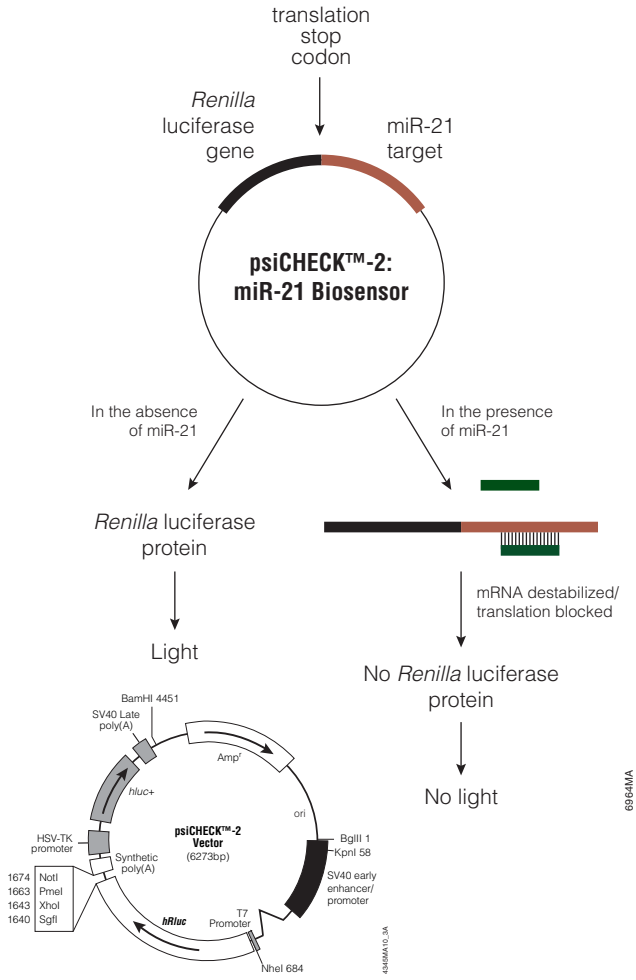

Figure 1. Detection of miR-21 activity using the psiCHECK™-2 Vector. miR-21 target sequence was cloned into the multiple cloning region of the psiCHECK™-2 Vector.

Table 1. Relative miR-21 microRNA Expression Levels Among Several Human Cell Types.

Cell Line	Relative miR-21 Expression
HEK293 (kidney)	Low (3)
K-562 (chronic myelogenous leukemia)	Low to moderate (3)
HeLa (cervical carcinoma)	High (4)
MCF-7 (breast adenocarcinoma)	High (3)

Our goal was to determine if *Renilla* luciferase expression would follow the same trends as those reported for HEK293, K-562, HeLa, and MCF-7 cells. We also wanted to determine if the psiCHECK™-2 Vector could report a known miR-21 target (miR-21 mismatch; 5). Complementary oligonucleotides containing the exact miR-21 sequence or a mismatched miR-21 target (5) were generated with flanking partial SgfI and PmeI restriction sites (Table 2). A restriction site not found in the psiCHECK™-2 Vector was also included to allow easy insert screening (EcoICRI). Annealed oligonucleotides were cloned into the psiCHECK™-2 Vector that had been digested previously with SgfI and PmeI. The psiCHECK™-2:miR-21 and miR-21 mismatch constructs were purified using the PureYield™ Plasmid Maxiprep System and confirmed by sequencing. Note that the biosensor was generated by cloning the reverse complement of the miRNA sequence downstream of the *Renilla* luciferase reporter gene. In this orientation, miRNA within the cells would then bind the fusion transcript and regulate translation.

Detecting miR-2l in Cell Lines

Amaxa Nucleofector® technology was used for all biosensor transfections. The Nucleofector® technology is a highly efficient nonviral method that relies on unique electrical charges and cell line-specific solutions for rapid transfections (www.amaxa.com). All analyses were done 18–24 hours after transfection using the Dual-Glo™ Luciferase Assay System and the GloMax® 96 Microplate Luminometer.

As shown in Figure 2, addition of the miR-21 target sequence in the 3′ UTR of the *Renilla* luciferase transcript significantly affected expression in all cell lines tested. The biggest effect was seen in cell lines previously reported to have high miR-21 levels [HeLa (2) and MCF-7 (1)]. Based on Northern blot, K-562 and HEK293 cells have less miR-21 than MCF-7, and HEK293 cells have the least (1). This trend is also seen in the luciferase data, and supports the use of psiCHECK™-2 as an miRNA biosensor. The internal firefly luciferase control normalized for differences between experimental replicates and allowed detection of statistically significant differences, even using different passage numbers of cells.

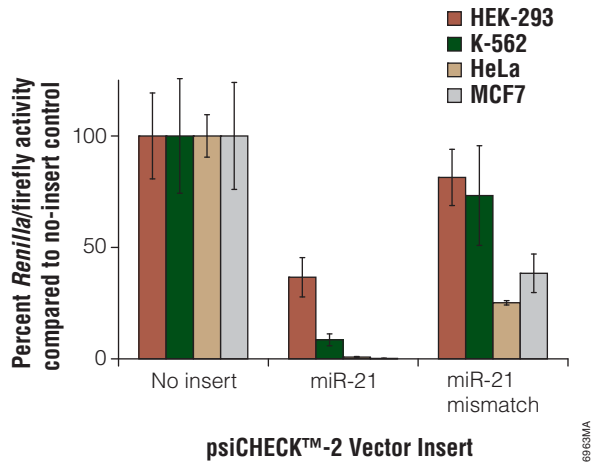

Figure 2. Detection of miR-21 activity using the psiCHECK™-2:miR-21 biosensor. Eighteen to 24 hours after transfection with the indicated psiCHECK™-2 construct, cells were analyzed for luciferase activity using the Dual-Glo™ Luciferase Assay System and the GloMax® 96 Microplate Luminometer. Normalized *Renilla* luciferase activity (*Renilla* luciferase/firefly luciferase) for each construct was compared to the psiCHECK™-2 no-insert control. Data are the average of three separate transfections performed on different days with different passage numbers of cells. For each transfection, luciferase activity was averaged from 2–4 replicates.

miRNA targets in the 3′ UTRs of genes contain mismatches to the miRNAs that regulate them. We tested psiCHECK™-2 Vector as a screening tool for targets of miR-21 activity using a mismatched miR-21 target sequence (5). Figure 2 shows that the miR-21 mismatch affects *Renilla* luciferase expression within those cells that have high miR-21 levels.

Summary

We demonstrated use of the psiCHECK™-2 Vector as a biosensor for miRNA activity and as a screening tool for miRNA targets. By cloning the inverted complement of miR-21 into the psiCHECK™-2 Vector, we were able to look at relative differences in miR-21 activity between cell lines. By cloning an miR-21 mistmatch target into the psiCHECK™-2 Vector, we measured the effect of the target on reporter expression in high versus low miR-21-containing cell lines.

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Table 2. Oligonucleotides used for Cloning into the psiCHECK™-2 Vector.

	Oligonucleotides (sequences identical or complementary to miR-21 are capitalized)
psiCHECK™-2:miR-21	Oligo 1: (5′) cgcagtagagctctagt TCAACATCAGTCTGATAAGCTA gttt (3′)
	Oligo 2: (5′) aaac TAGCTTATCAGACTG ATGTTGA actagagctctactgcgat (3′)
psiCHECK™-2:miR-21 mismatch	Oligo 1: (5′) cgcagtagagctctagt TCAACATCAGaaGATAAGCTA gttt (3′)
	Oligo 2: (5′) aaac TAGCTTATCttCTGATGTTGA actagagctctactgcgat (3′)

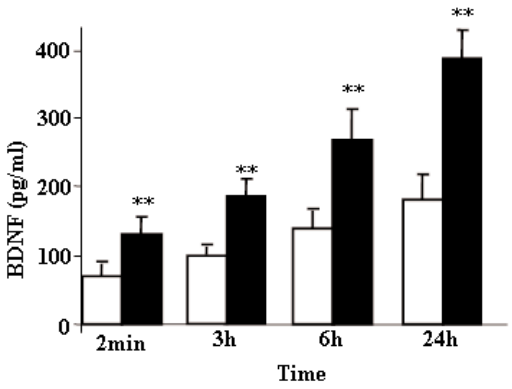

Figure 1. NMDA evokes the release of BDNF in hippocampal medium. Hippocampal cultures were exposed for 2 minutes, 3 hours, 6 hours and 24 hours to medium alone (white bars) or to medium containing NMDA (50 µM, black bars) beginning on DIV 8. Medium (2 ml) was collected, concentrated to 100 µl, and assayed by the ELISA two-site immunoassay. Data are expressed as the mean ± SD; n = 5; **p < 0.05 versus medium alone by Anova. Figure and legend reprinted with the kind permission of A. Marini and *J. Neurochemistry* from reference 1.

References

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Protocol

BDNF E_{max}® ImmunoAssay System Technical Bulletin #TB257 (www.promega.com/tbs/tb257/tb257.html)

Ordering Information

Product	Size	Cat.#
BDNF E_{max}™ ImmunoAssay System	2 × 96 wells	G7610
	5 × 96 wells	G7611

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The psiCHECK™ Vectors allow screening for the translational effects of not just miRNA targets but any 3´ UTR sequence. By appending the 3´ UTR of interest to the *Renilla* luciferase gene, luciferase activity can be used as a marker for 3´ UTR regulation. Inclusion of the firefly luciferase reporter in the psiCHECK™-2 Vector has the additional benefit of serving as the internal control to allow you to improve day-to-day reproducibility. Use of luciferase reporters allows easy screening of sequences, cell lines, and growth and stimulation conditions directly in cell culture wells.

References

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Acknowledgements

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Ordering Information

Product	Size	Cat.#
psiCHECK™-2 Vector	20 µg	C8021

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[b]U.S. Pat. No. 5,670,356.
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