Using Bioluminescent Reporter Genes to Optimize shRNA Target Sites for RNAi of the *bcr/abl* Gene

By Natalie Betz, Ph.D., Promega Corporation

Abstract

Not all shRNAs are equally effective at suppressing target gene expression in mammalian cells. Therefore it is important to quickly and easily identify the shRNA sequence that is the most effective at inducing RNA interference for a particular target gene sequence. The psiCHECK™-2 Vector, which contains two luminescent reporter genes, was used to identify the most effective shRNA for inducing RNAi of the bcr/abl *gene. The seven shRNAs tested were generated using the T7 RiboMAX™ Express RNAi System.*

The psiCHECK™-2 Vector provided a quick and easy approach for screening seven shRNAs against the *bcr/abl* gene.

Introduction

In mammalian cells, RNA interference (RNAi) induces a decrease in gene expression of a particular target by short interfering RNAs (siRNAs) or short hairpin RNAs (shRNAs). These siRNAs interact with the RNA-induced silencing complex (RISC), where they function as templates for recognition and cleavage of complementary mRNA targets, thus reducing protein levels (reviewed in reference 1).

Not all siRNAs or shRNAs generated for a target gene are equally effective in silencing that gene in mammalian cells. It is important to quickly and easily identify the shRNA sequence that is the most effective inhibitor of target gene expression. Often, screening for target site selection involves semi-quantitative, time-consuming methods that are not easily modified for rapid and simultaneous screening of multiple sequences.

The psiCHECK™-2 Vector[a–f] provides a quantitative and rapid approach for the optimization of RNAi site selection (2). The vector enables changes in expression of a target gene to be determined by monitoring the activity of a fused *Renilla* luciferase reporter gene. A target gene of interest is cloned downstream of the stop codon for the *Renilla* luciferase gene, such that a hybrid mRNA is expressed (but no hybrid protein). Initiation of RNAi toward the target gene results in the cleavage and subsequent degradation of the fusion mRNA including the *Renilla* luciferase RNA sequence. This results in a decrease in *Renilla* luciferase activity that can be easily monitored as an indicator of the RNAi effect. The presence of a second reporter gene (firefly luciferase) in the psiCHECK™-2 Vector allows for normalization of transfection efficiency. *Renilla* and firefly luciferase activity can be measured very easily using the Dual-Glo™ Luciferase Assay System.

To determine an optimal shRNA sequence for the *bcr/abl* target gene, seven different shRNAs were synthesized using the T7 RiboMAX™ Express RNAi System[g–i], which utilizes in vitro transcription and double-stranded oligonucleotide DNA templates (3). The *bcr/abl* gene represents a reciprocal translocation between chromosomes 9 and 22, resulting in the cytogenetically distinct Philadelphia chromosome, observed in 90–95% of patients with chronic myelogenous leukemia (4). This target was selected for RNAi given its clinical relevance.

Generation of the psiCHECK™-2-*bcr/abl* Construct

A 200bp region of the *bcr/abl* mRNA was amplified by RT-PCR[j] using the Access RT-PCR System with total RNA from K562 cells, a human CML cell line, which is positive for the Philadelphia chromosomal translocation (4; GenBank® accession# M30829). This product spans the junction region between the *bcr* and *abl* genes of the hybrid mRNA. The amplification product contained *Sgf* I and *Pme* I restriction sites included in the forward and reverse primers, respectively (forward = 5′-GTCA**GCGATCGC**GGAGCTGCAGATGCTGACCAAC; reverse = 5′-GATC**GTTTAAAC**TCAGACCCTGAGGCTCAAAGTC). The RT-PCR product was then cloned into the psiCHECK™-2 Vector also digested with *Sgf* I and *Pme* I enzymes. The psiCHECK™-2-*bcr/abl* plasmid was purified using the PureYield™ Plasmid Midiprep System.

Synthesis of Seven Different shRNAs to the *bcr/abl* mRNA

shRNAs were synthesized using the T7 RiboMAX™ Express RNAi System and seven different double-stranded oligonucleotide templates. These seven shRNAs spanned the 200bp sequences included in the psiCHECK™-2-*bcr/abl* construct. In addition, a shRNA targeting *Renilla* luciferase was also generated as a positive control for RNAi. The sequences of the shRNAs are listed in Table 1. The shRNAs have the general structure of: 5′-sense target sequence-loop-antisense target sequence-UU-3′.

Table 1. shRNA Sequences.

Target Site	shRNA Sequence
bcr/abl site 1	5′-GCUGCAGAUGCUGACCAAC-uucaagaga-GUUGGUCAGCAUCUGCAGC-UU-3′
bcr/abl site 2	5′-GUGAAACUCCAGACUGUCC-uucaagaga-GGACAGUCUGGAGUUUCAC-UU-3′
bcr/abl site 3	5′-GAAACUCCAGACUGUCCAC-uucaagaga-GUGGACAGUCUGGAGUUUC-UU-3′
bcr/abl site 4	5′-GUUUCUGAAUGUCAUCGUC-uucaagaga-GACGAUGACAUUCAGAAAC-UU-3′
bcr/abl site 5	5′-GUAGCAUCUGACUUUGAGC-uucaagaga-GCUCAAAGUCAGAUGCUAC-UU-3′
bcr/abl site 6	5′-GCAUCUGACUUUGAGCCUC-uucaagaga-GAGGCUCAAAGUCAGAUGC-UU-3′
bcr/abl site 7	5′-GACUUUGAGCCUCAGGGUC-uucaagaga-GACCCUGAGGCUCAAAGUC-UU-3′
Renilla luciferase	5′-GCUGGACUCCUUCAUCAAC-uucaagaga-GUUGAUGAAGGAGUCCAGC-UU-3′

Determination of the Most Effective shRNA for RNAi

To determine which *bcr/abl* shRNA was most effective at inducing degradation of the mRNA and thus inducing RNA interference, Chinese hamster ovary cells (CHO) were transfected with the psiCHECK™-2-*bcr/abl* plasmid as well as the various shRNAs directed against either the *bcr/abl* portion of the hybrid mRNA or the *Renilla* portion of the hybrid mRNA. CHO cells were plated at 4×10^4 cells/500µl growth medium/well in 24-well tissue culture plates. Following incubation overnight at 37°C (5% CO_2), the cells were approximately 40–50% confluent.

The cells were first transfected with the psiCHECK™-2-*bcr/abl* reporter vector using TransFast™ Transfection Reagent. A transfection mix containing 200µl Opti-MEM® medium (Invitrogen), 3µl of TransFast™ Reagent, and 0.5µg psiCHECK™-2-*bcr/abl* plasmid DNA was prepared as described in Technical Bulletin #TB260. The growth medium was removed, and 200µl of the transfection mix added to each well and incubated at 37°C for 1 hour. Following this incubation, 1ml of complete growth medium was then added to each well and the cells incubated overnight at 37°C.

The next morning, shRNA transfection mixes were prepared with 200µl Opti-MEM® medium, 1.52µl CodeBreaker™ siRNA Transfection Reagent and 200ng shRNA as described in Technical Bulletin #TB326. The growth medium was removed from the cells and replaced with 400µl fresh complete growth medium. An aliquot of the shRNA transfection mix (200µl) was then added to each of triplicate wells and incubated overnight at 37°C. This delayed transfection method (plasmid first, shRNA later) was found to produce more RNAi as compared to simultaneous transfection of both the plasmid DNA and the shRNAs (data not shown).

The Dual-Glo™ Luciferase Assay System was used to measure *Renilla* luciferase and firefly luciferase reporter activity. The ratio of *Renilla* luciferase activity to firefly luciferase activity for each transfected shRNA (or the no-shRNA control) was calculated, and the percent inhibition of this ratio (as compared to the no-shRNA control) was determined. The results are shown in Figure 1 and demonstrate that the *bcr/abl* site 7 shRNA was as effective at inducing degradation of the hybrid *Renilla/bcr/abl* mRNA as the *Renilla* shRNA, with both showing approximately 90% inhibition of the *Renilla* luciferase activity signal (when normalized for transfection efficiency using the firefly luciferase activity signal). Thus the psiCHECK™-2 Vector serves as an easy and useful screening tool for selection of an optimal shRNA or siRNA target site.

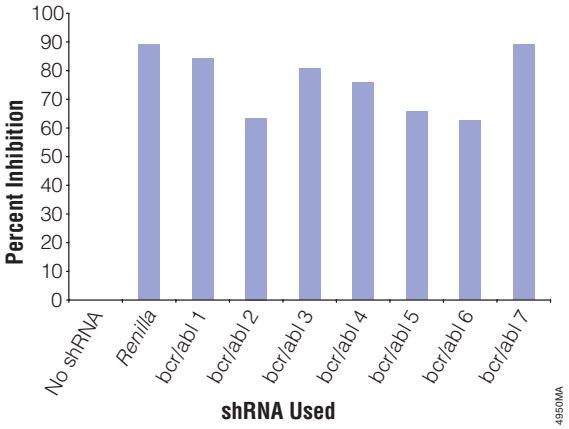

Figure 1. Inhibition of *Renilla* luciferase activity by various shRNAs. Transfections were performed using the psiCHECK™-2-*bcr/abl* construct and shRNAs directed against *Renilla* or *bcr/abl* and compared to the no-shRNA control. *Renilla* and firefly luciferase was measured using the Dual-Glo™ Luciferase Assay. Medium was removed from each well and replaced with 300µl fresh complete growth medium. Reconstituted Firefly Luciferase Reagent (300µl) from the Dual-Glo™ Luciferase Assay System was added to each well and the plate incubated for 5 minutes at room temperature with shaking. An aliquot from each well (100µl) was then transferred to a white 96-well plate and luminescence measured in a Berthold EG&G plate reading luminometer (2-second read time; RLU factor = 1.0). Prepared *Renilla* Luciferase Reagent from the Dual-Glo™ Luciferase Assay System (50µl) was then added to each well, and the plate was incubated for 5 minutes at room temperature with shaking. Light output was then measured as for firefly luciferase. The results are the average of triplicate wells for each shRNA.

Conclusions

The use of the dual-reporter psiCHECK™-2 Vector allowed for the quick and easy screening of seven different shRNAs to *bcr/abl* to determine which target site was most effective at inducing degradation of the mRNA. This *bcr/abl* target site shRNA can now be used for the induction of RNAi in K562 cells, which are much more difficult to transfect as compared to CHO cells and would be much more difficult to use for the screening stage. In general, the efficacy of different shRNAs to a particular target sequence in the

psiCHECK™ Vector hybrid mRNA is also observed on the endogenous target mRNA.

The psiCHECK™ Vectors can also be used to monitor RNAi kinetics using the EnduRen™ Live Cell Substrate (2) as compared to the endpoint assays performed in this study with the Dual-Glo™ Luciferase Assay System.

References

1. Betz, N. (2003) *Promega Notes* **83**, 33–6.
2. Vidugiriene, J. *et al.* (2004) *Promega Notes* **87**, 2–6.
3. Betz, N. and Worzella, T. (2003) *Promega Notes* **85**, 15–8.
4. Tbakhi, A. *et al.* (1998) *Am. J. Clin. Pathol.* **109**, 16–23.

Protocols

- *psiCHECK™ Vectors Technical Bulletin* #TB329, Promega Corporation. (www.promega.com/tbs/tb329/tb329.html)
- *T7 RiboMAX™ Express RNAi System Technical Bulletin* #TB316, Promega Corporation. (www.promega.com/tbs/tb316/tb316.html)
- *TransFast™ Transfection Reagent Technical Bulletin* #TB260, Promega Corporation. (www.promega.com/tbs/tb260/tb260.html)
- *CodeBreaker™ siRNA Transfection Reagent Technical Bulletin #*TB326, Promega Corporation. (www.promega.com/tbs/tb326/tb326.html)
- *Dual-Glo™ Luciferase Assay System Technical Manual* #TM058, Promega Corporation. (www.promega.com/tbs/tm058.tm058.html)
- *Access RT-PCR System Technical Bulletin* #TB220, Promega Corporation. (www.promega.com/tbs/tb220/tb220.html)
- *PureYield™ Plasmid Midiprep System Technical Manual* #TM253, Promega Corporation. (www.promega.com/tbs/tm253/tm253.html)

Natalie Betz, Ph.D.
Applications Scientist

Ordering Information

Product	Size	Cat. #
psiCHECK™-1 Vector	20µg	C8011
psiCHECK™-2 Vector	20µg	C8021
T7 RiboMAX™ Express RNAi System	50 × 20µl reactions	P1700
Dual-Glo™ Luciferase Assay System	10ml	E2920
	100ml	E2940
	10 × 100ml	E2980
TransFast™ Transfection Reagent	1.2mg	E2431
CodeBreaker™ siRNA Transfection Reagent	0.4ml	E5052
	1.0ml	E5053
Access RT-PCR System*	20 reactions	A1260
	100 reactions	A1250
	500 reactions	A1280
PureYield™ Plasmid Midiprep System	25 preps	A2492
	100 preps	A2495

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

[c] The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673. A license (from Promega for research reagent products and from The Regents of the University of California for all other fields) is needed for any commercial sale of nucleic acid contained within or derived from this product.

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[h] U.S. Pat. Nos. 4,966,964, 5,019,556 and 5,266,687, Australian Pat. Nos. 616881 and 641261 and other pending and issued patents, which claim vectors encoding a portion of human placental ribonuclease inhibitor, are exclusively licensed to Promega Corporation.

[i] U.S. Pat. No. 5,552,302, Australian Pat. No. 646803 and other patents.

[j] The PCR process is covered by patents issued and applicable in certain countries*. Promega does not encourage or support the unauthorized or unlicensed use of the PCR process.

* In Europe, effective March 28, 2006, European Pat. Nos. 201,184 and 200,362, will expire. In the U.S., the patents covering the foundational PCR process expired on March 29, 2005.

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