pGreenFire 2.0 Pathway Reporter Lentivector & Virus

Cat # TR4XXPA/VA-P

User Manual

Storage:
TR4XXPA-P at -20˚C
TR4XXVA-P at -70˚C

Version 1
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Contents

Product Description

Monitor signal transduction in real time with our re-engineered pGreenFire 2.0 Lentivectors

Eukaryotic gene expression is regulated by a wide variety of developmental and environmental stimuli. First, an extracellular signaling molecule binds to a specific receptor. The signal is then transmitted through a series of molecular cascades, which activate or deactivate specific transcription factors that regulate gene expression. The expression of any given gene is controlled by multiple transcription factors, which in turn are modulated by multiple signal transduction pathways. Many of these signal transduction pathways converge at transcription factors that bind to specific transcriptional response elements found in the promoters of various genes and modulate the transcription of these genes. The activation of a signal transduction pathway (e.g. by growth factors, drugs, etc.) can therefore be monitored by the expression level of the reporter gene controlled by a promoter containing these response elements.

SBI has upgraded our popular pGreenFire signaling pathway reporter lentivectors with a design that leads to more reliable generation of stable cell lines. We've also swapped in the red firefly luciferase reporter (rFLuc), which opens up the possibility of performing a dual-spectral luciferase assay and delivers greater sensitivity for in vivo applications than conventional luciferase.

With the pGreenFire 2.0 Reporter Lentivector & Virus (pGF2-XXX-rFluc-T2A-GFP-mPGK-Puro), the core reporter functionality is similar to the original pGreenFire lentivector— transcriptional response elements (TREs) are placed upstream of a minimal CMV promoter (mCMV) which together drive co-expression of rFLuc and GFP in response to specific transcription factor activity. The result is the ability to quantitatively measure specific transcription activity using both fluorescence and luciferase activity.

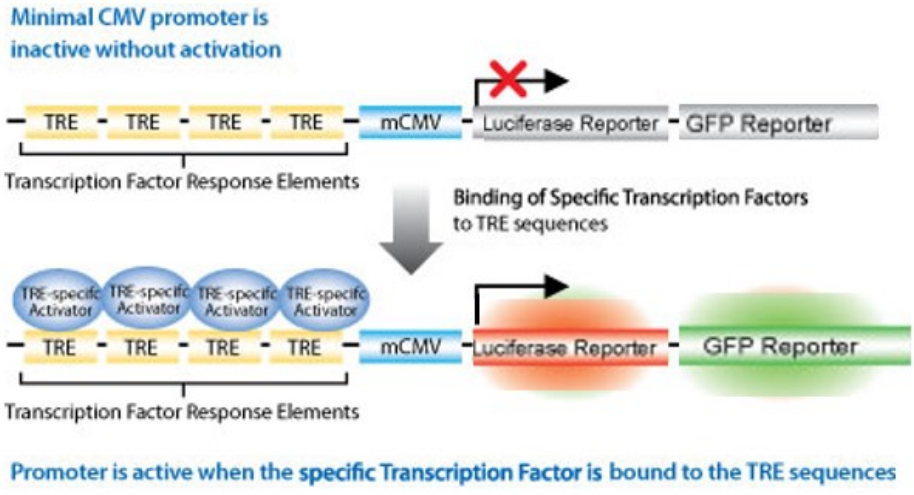


Figure 1. Quantitatively measure trsnacription activity using both fluorescence and luciferase activity.

What makes our next-gen pGreenFire 2.0 vectors even better than other TRE reporter vectors is the smart design, which adds in a constitutive selection cassette for stable cell line generation while minimizing interference with the upstream TRE. By using a weak/moderate mPGK promoter to drive the antibiotic selection marker (puromycin resistance) and carefully arranging the conditional reporter genes, the selection marker is reliably expressed without compromising conditional expression of rFLuc and GFP.

As with our original pGreenFire1 vectors, all pGreenFire 2.0 lentivectors leverage our reliable lentivector technology and save you time with pre-built signal transduction pathway reporters that come as ready-to-transduce pre-packaged lentivirus and plasmid that can be transfected into the lentivirus producing system of your choice*.

- Sort responsive cells with GFP
- Measure activity with red firefly luciferase
- Leverage SBI's highly-regarded lentivectors
- Create stable signaling pathway reporter cell lines
- Introduce reporters into difficult-to-transfect cell types, including primary and non-dividing mammalian cell lines.

***Please note** that these vectors only function properly when transduced. Transfection keeps the constitutive RSV promoter intact, leading to nonspecific expression of the reporter genes.

Safety Guidelines

SBI's pGreenFire 2.0 lentivectors are based on the traditional HIV vector backbone. To address biosafety issues, SBI uses a third generation HIV lentiviral vector. (Dull, et.al., 1998, Miyoshi, et.al., 1998, Zufferey, et.al., 1999, Ramezani, et.al. 2000). SBI's lentiviral vectors are efficient gene transfer vehicles, as used for research applications, because of their stable integration in non-dividing and dividing cells and long-term transgene expression. SBI's HIV-based lentivector systems are designed to maximize their biosafety features, which include:

- A deletion in the enhancer of the U3 region of 3'ΔLTR ensures self-inactivation of the lentiviral construct after transduction and integration into genomic DNA of the target cells.
- Upon integration into the genome, the 5' LTR promoter is inactivated, which prevents formation of replication-competent viral particles.
- The RSV promoter in HIV-based vectors, upstream of 5'LTR in the lentivector, allow efficient Tat-independent production of viral RNA, reducing the number of genes from HIV-1 that are used in this system.
- The number of lentiviral genes necessary for packaging, replication and transduction is reduced to three (gag, pol, rev).

- The corresponding proteins are expressed from different plasmids that lack packaging signals. The packaging plasmids share no significant homology to any of the expression lentivectors, the pVSV-G expression vector, or any other vector, to prevent generation of recombinant replication-competent virus.
- None of the HIV-1 genes (gag, pol, rev) are present in the packaged viral genome, as they are expressed from separate plasmids lacking packaging signal. Therefore, the lentiviral particles generated are replication-incompetent.
- For pGreenFire 2.0Zeo constructs, produced pseudoviral particles will carry only a copy of your cell specific promoter sequence.

The choice of SBI's lentiviral system for experimental studies is driven by functional considerations, including increased productivity and transduction efficiency. The design of SBI's biosafe vectors has benefited researchers allowing them to conduct experimental studies with lower risk. Currently, SBI's vectors combine improved safety features (that decrease the risk of recombination and vector mobilization) with increased transduction efficiency.

Despite the above safety features, use of HIV-based vectors falls within NIH Biosafety Level 2 criteria due to the potential biohazard risk of possible recombination with endogenous viral sequences to form self-replicating virus, or the possibility of insertional mutagenesis. For a description of laboratory biosafety level criteria, consult the Centers for Disease Control Office of Health and Safety Web site at

http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4s3.htm

It is also important to check with the health and safety guidelines at your institution regarding the use of lentiviruses and to always follow standard microbiological practices, which include:

- Wear gloves and a lab coat when handling the lentiviral vectors, pseudoviral particles, or transduced cells.
- Always work with pseudoviral particles in a Class II laminar flow hood.
- Perform all procedures carefully to minimize splashes, spills or the production of aerosols.
- Decontaminate work surfaces at least once a day or after any spill of viable material.
- Decontaminate all cultures, stocks, and other regulated wastes before disposal by an approved decontamination method such as autoclaving. Materials to be decontaminated outside of the immediate laboratory area should be placed in a durable, leakproof, properly marked (biohazard, infectious waste) container and sealed for transportation from the laboratory.

List of Components

TR4XXXPA-P : pGreenFire 2.0 reporter plasmid (pGF2-XXX-rFluc-T2A-GFP-mPGK-Puro), 10ug

TR4XXVA-P: pGreenFire 2.0 reporter virus (pGF2-XXX-rFluc-T2A-GFP-mPGK-Puro,)>2 x10^6 IFUs

Note: The exact titer of the virus is supplied on the lot specific Product Analysis Certificate supplied with the product. Please aliquote virus at first use to avoid repeated freeze thaws.

Additional Required and Optional Equipment Not Included in Kit

- LB Agar and Broth with Ampicillin
- One Shot OmniMAX2 competent cells (Invitrogen, Cat # C8540-03 or other RecA- E. coli competent cells)
- Dulbecco's Modified Eagle's Medium (D-MEM) high glucose with sodium pyruvate and glutamine (Invitrogen, Cat. # 11995073)
- Fetal Bovine Serum (Invitrogen, Cat. # 16000036)
- Penicillin/Streptomycin (Invitrogen, Cat. # 15070063)
- Trypsin-EDTA (Sigma, Cat. # T3924)
- TransDux™ (SBI, Cat. # LV850A-1)
- Millex-HV 0.45 □m PVDF filters (Millipore, Cat. # SLHVR25LS)
- Tissue Culture Plates and Related Tissue Culture Supplies
- 293TN Human Kidney Producer Cell Line (SBI, Cat. # LV900A-1)

Protocol

Key Terms

MOI (multiplicity of infection): The ratio of infectious pseudoviral particles (ifu) to the number of cells being infected. IFU/ # cells = MOI

IFU/ml (infectious units per ml): The relative concentration of infection-competent pseudoviral particles. Also called pseudoviral titer.

Transduction Efficiency: The average copy number of expression constructs per genome of target cell in the infected population.

General Comments

To ensure optimal results, follow these general guidelines during your experiments:

pGF1 2.0-CMV Positive Control Lentivector and Virus, TR410PA-P and TR410VA-P: This plasmid should be used to estimate transduction efficiency of the lentiviral expression construct into target cells, select the cell type with highest infection efficiency, and to optimize the transduction protocol. Moreover, the presence of GFP-positive

cells indicates that the lentiviral construct can be efficiently expressed in your target cells from the CMV promoter. The construct can also be used for calibration of FACS machine for maximum intensity of expression.

pGF 2.0-mCMV Negative Control Lentivector and Virus, TR411PA-P and TR411VA-P: Negative control construct which can be used to transduce target cells under the conditions optimized for the positive control pGF 2.0-CMV construct and determine "background" of GFP fluorescence of target cells with a non-activated CMV promoter.

The transduction efficiency of the pGreenFire 2.0 Packaged Reporter Construct may vary significantly for different cells and experimental conditions. In order to optimize transduction conditions, we recommend that you use HT1080 (or similar) cells as a positive control in parallel with your target cells and use prepackaged pGF 2.0-CMV (TR04110VA-P1) from SBI. To determine the desired multiplicity of infection (MOI) appropriate for your target cells, you should do several transductions with packaged pGreenFire 2.0 pseudoviral particles at different MOI's (e.g. from 0.1 to 5). Results of these test transductions should be used to determine an optimal MOI that yields the optimal percentage of infected cells based on the percentage of cells expressing the GFP marker. Note that some cell types, such as primary cells may be resistant to infection regardless of the MOI.

Expression of the pGreenFire 2.0 Reporter can be measured directly at about 48-72 hours after transduction. At this time, pGreenFire1 2.0 constructs are integrated into the genomic DNA resulting in stably transduced reporter cell lines. Reporter cells can be cloned in order to obtain a uniform population of the GreenFire cell line. Some cells may express the reporter construct in 80-90% of the cells after transduction at MOI of 1-2. For these "easy-to-transduce" cells, most biological assays can be performed at 48-72 hours after transduction. However, some primary cells may only express the construct in 10-50% of cells, even when transduced at high MOI's. For these "difficult-to-transduce" cells, it is probably desirable to select the cells stably expressing the construct by FACS or clonal selection for experimental assays.

Due to the pathway specificity of the pGreenFire2.0 reporter, GFP and luciferase expression is only expected to occur in cell types that express the appropriate transcription factors that bind to the TRE regions of the promoter.

pGreenFire 2.0 Plasmid Preparation

1. Transformation of pGreenFire1 lentivector reporter constructs into competent cells: If you have purchased the pGreenFire1 reporter construct as a plasmid, you will need to produce enough for packaging into virus. We recommend using E.coli that are RecA- competent cells. (Invitrogen One Shot OmniMAX2 Competent Cells C8540-03) Please follow the protocol according to the manufacturer's instructions. The transformed E.coli can be grown at 370°C on LB agar with ampicillin overnight.
2. Plasmid Purification: Lentivector constructs must be grown in liquid culture (LB with Ampicillin) at 30°C. E. coli transformed with lentivector constructs seem to expel the plasmid if grown at 37°c.

SBI recommends the PureLink™ Hi Pure Plasmid Filter Purification Kit from Invitrogen (Cat. # K2100-14) for purification of lentivector plasmids.

Production of pGreenFire 2.0 packaged virus

Please refer to the "Lentivector Expression Systems: Guide to Packaging and Transduction of Target Cells" manual for a full description of how to package pGreenFire1 into VSV-G pseudotyped viral particles.

Transduction of pGreenFire 2.0 into target cells

The following protocol describes the general procedure for the transduction of the pGreenFire1 Reporter Constructs packaged in pseudotyped viral particles into HT1080 cells. This protocol assumes that you will use these guidelines in order to perform transduction of your target cells in parallel using HT1080 cells as a positive control and can be used as a starting point for the optimization for transduction of your particular cell-type.

Day 1

1. Plate 50,000 cells per well in a 24 well plate in cell culture medium.

Day 2

2. Cells should be between 50 to 70% confluent. Aspirate medium from cells.

3. Combine culture medium with TransDux to a 1X final concentration. (For example, add 2.5 µl of TransDux to 500 µl culture medium and then transfer to each well.)

4. Add virus to each well and swirl to mix. (Optional: Add increasing amounts of virus to different wells at varying MOIs (5, 10 and 20, etc.) to optimize the transduction.

Day 5

5. 72 hours post transduction, the viral genome will be integrated into the host cell genome. Look at the cells for reporter expression if the viral construct has a reporter like GFP.

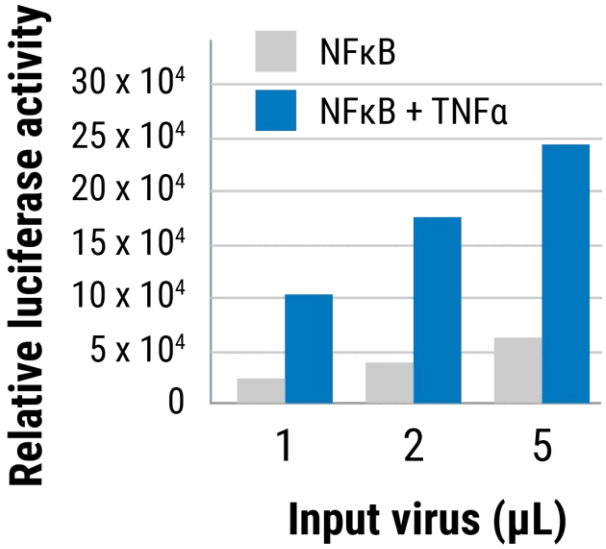
6. Aspirate off medium. Wash each well with PBS.

7. To establish stabile cell lines, you can now FACs sort for GFP or RFP positive cells. If using an antibiotic selection marker, you can begin your selection procedure.

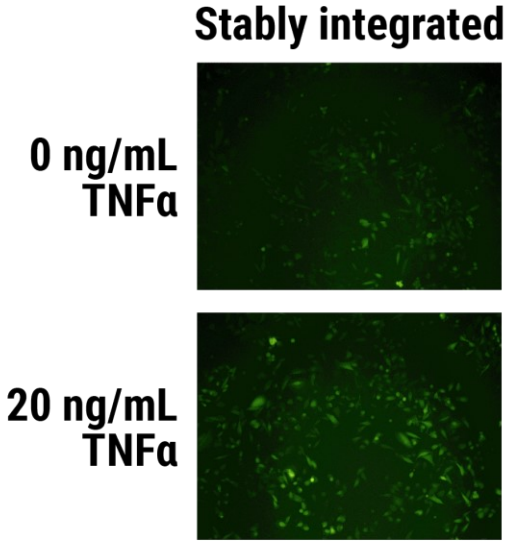
Example Data and Applications

The pGreenFire 2.0 NFκB Reporter efficiently and quantitatively reports on NFκB activity in MDA-MB-213 cells. Relative luciferase activity (A) and GFP activity (B) both increase in response to TNFα, and NFκB inducer. (C) Like all pGreenFire 2.0 lentivectors, the pGreenFire 2.0 NFκB Reporter contains an mPGK-Puro cassette to streamline creation of stable reporters integrated into the cell lines of your choice.

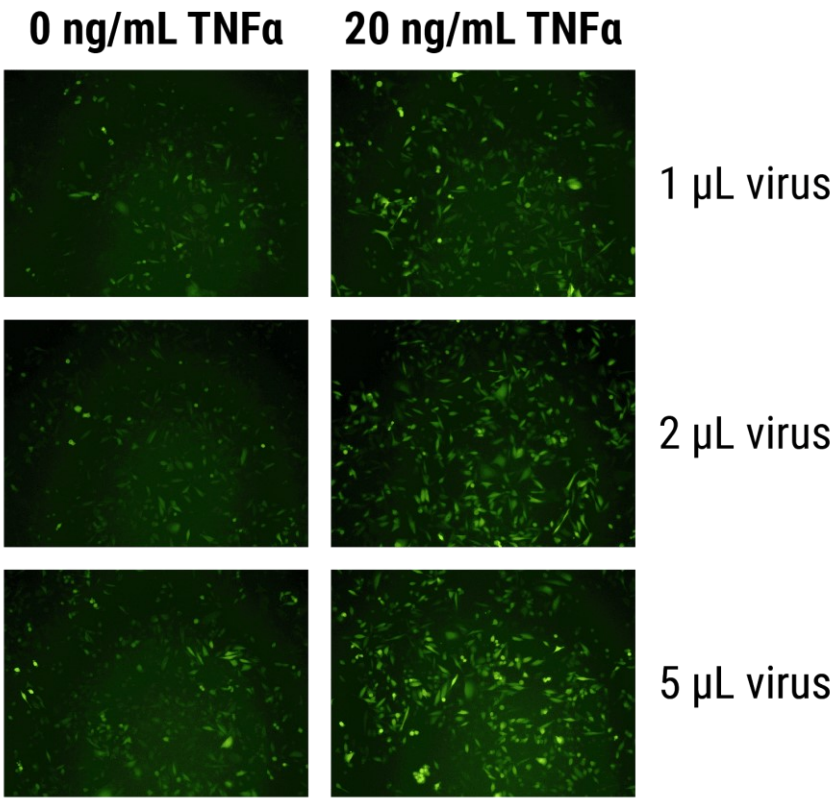
A.


Relative luciferase activity
30 x 10⁴
25 x 10⁴
20 x 10⁴
15 x 10⁴
10 x 10⁴
5 x 10⁴
0
NFκB
NFκB + TNFα
1
2
5
Input virus (µL)

C.


Stably integrated
0 ng/mL TNFα
20 ng/mL TNFα

B.


0 ng/mL TNFα
20 ng/mL TNFα
1 µL virus
2 µL virus
5 µL virus

References

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