6042 Cornerstone Court W, Ste B
San Diego, CA 92121
Tel: 1.858.829.3082
Fax: 1.858.481.8694
Email: info@bpsbioscience.com

Data Sheet

SBE Reporter Kit (TGFβ/SMAD signaling pathway)
Catalog #: 60654

Background

The transforming growth factor beta (TGFβ) signaling pathway is involved in a diverse range of cell processes such as differentiation, cell cycle arrest, and immune regulation. TGFβ signaling has been linked to cardiac disease, cancer, Alzheimer's and other human diseases. TGFβ proteins bind to receptors on the cell surface, initiating a signaling cascade that leads to phosphorylation and activation of SMAD2 and SMAD3, which then form a complex with SMAD4. The SMAD complex then translocates to the nucleus and binds to the SMAD binding element (SBE) in the nucleus, leading to transcription and expression of TGFβ/ SMAD responsive genes.

Description

The SBE Reporter kit is designed for monitoring the activity of TGFβ/SMAD signaling pathway in the cultured cells. The kit contains transfection-ready SBE luciferase reporter vector, which is a TGFβ pathway-responsive reporter. This reporter contains a firefly luciferase gene under the control of multimerized SBE responsive element located upstream of a minimal promoter. The SBE reporter is premixed with constitutively expressing *Renilla* luciferase vector that serves as internal control for transfection efficiency.

The kit also includes a non-inducible firefly luciferase vector premixed with constitutively expressing *Renilla* luciferase vector as negative control. The non-inducible luciferase vector contains a firefly luciferase gene under the control of a minimal promoter, without any additional response elements. The negative control is critical to determining pathway-specific effects and background luciferase activity.

Applications

- Monitor TGFβ signaling pathway activity.
- Screen activators or inhibitors of TGFβ/ SMAD signaling pathway.
- Study effects of RNAi or gene overexpression on the activity of TGFβ pathway.

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Components

Component	Specification	Amount	Storage
Reporter (Component A)	SBE luciferase reporter vector + constitutively expressing *Renilla* luciferase vector	*500 µl (60 ng DNA/ µl)*	-20°C
Negative Contol Reporter (Component B)	Non-inducible luciferase vector + constitutively expressing *Renilla* luciferase vector	*500 µl (60 ng DNA/ µl)*	-20°C

These vectors are ready for transient transfection. They are NOT SUITABLE for transformation and amplification in bacteria.

Materials Required but Not Supplied

- Mammalian cell line and appropriate cell culture medium
- 96-well tissue culture plate or 96-well tissue culture-treated white clear-bottom assay plate (Corning # 3610)
- Transfection reagent for mammalian cell lines [We use Lipofectamine™ 2000 (Invitrogen # 11668027). However, other transfection reagents work equally well.]
- Opti-MEM I Reduced Serum Medium (Invitrogen #31985-062)
- Dual Luciferase Assay System:
  Dual Luciferase (Firefly-Renilla) Assay System (BPS Cat. #60683): This system assays cells directly in growth medium. It can be used with any luminometer. Automated injectors are not required.
- Luminometer

Generalized Transfection and Assay Protocols

The following procedure is designed to transfect the reporter into HEK293 cells using Lipofectamine 2000 in a 96-well format. To transfect cells in different tissue culture formats, adjust the amounts of reagents and cell number in proportion to the relative surface area. If using a transfection reagent other than Lipofectamine 2000, follow the manufacturer's transfection protocol. Transfection conditions should be optimized according to the cell type and study requirements.

All amounts and volumes in the following setup are given on a per well basis.

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1. One day before transfection, seed HEK293 cells at a density of 30,000 cells per well in 100 µl of growth medium so that cells will be 90% confluent at the time of transfection. Incubate the plate at 37°C in a CO_2 incubator.

2. Next day, for each well, prepare complexes as follows:

   a. Dilute DNA mixtures in 15 µl of Opti-MEM I medium (antibiotic-free). Mix gently. Depending upon the experimental design, the DNA mixtures may be any of the following combinations:

   - **1 µl of Reporter** (component A); in this experiment, the control transfection is **1 µl of Negative Control Reporter** (component B).
   - **1 µl of Reporter** (component A) + experimental vector expressing gene of interest; in this experiment, the control transfections are: **1 µl of Reporte**r (component A) + negative control expression vector, **1 µl of Negative Control Reporter** (component B) + experimental vector expressing gene of interest, and **1 µl of Negative Control Reporter** (component B) + negative control expression vector.
   - **1 µl of Reporter** (component A) + specific siRNA; in this experiment, the control transfections are: **1 µl of Reporte**r (component A) + negative control siRNA, **1 µl of Negative Control Reporter** (component B) + specific siRNA, and **1 µl of Negative Control Reporter** (component B) + negative control siRNA.

   Note: we recommend setting up at least triplicates for each condition, and prepare "master mix" of transfection cocktail for multiple wells to minimize pipetting errors.

   b. Mix Lipofectamine 2000 gently before use, then dilute 0.35 µl of Lipofectamine 2000 in 15 µl of Opti-MEM I medium (antibiotic-free). Incubate for 5 minutes at room temperature.

   Note: Prepare this dilution cocktail in volumes sufficient for the whole experiment.

   c. After the 5-minute incubation, combine the diluted DNA with diluted Lipofectamine 2000. Mix gently and incubate for 25 minutes at room temperature.

3. Add the 30 µl of complexes to each well containing cells and medium. Mix gently by tapping the plate.

4. Incubate cells at 37°C in a CO_2 incubator. After ~24 hours of transfection, change medium to fresh medium. ~48 hours after transfection, perform the Dual Luciferase Assay System following the protocol on the BPS data sheet (BPS Cat. #60683).

To study the effect of activators / inhibitors on the TGFβ pathway, treat the cells with test activator/inhibitor after ~24 hours of transfection. Perform dual luciferase assay ~48 hours after transfection.

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Sample protocol to determine the dose response of HEK293 cells transfected with SBE reporter to human TGFβ1

Additional materials required for this experiment

- Human TGFβ1 (BPS Bioscience #90900-1)
- HEK293 growth medium: MEM/EBSS (with L-glutamine) (Hyclone #SH30024.01) + 10% FBS, 1% non-essential amino acids, 1 mM Na pyruvate + 1% Pen/Strep
- HEK293 assay medium: MEM/EBSS (with L-glutamine) (Hyclone #SH30024.01) + 0.5% FBS, 1% non-essential amino acids, 1 mM Na pyruvate + 1% Pen/Strep
- 96-well tissue culture-treated white clear-bottom assay plate (Corning # 3610)
- Dual Luciferase (Firefly-Renilla) Assay System (BPS Cat. #60683)

1. One day before transfection, seed HEK293 cells at a density of 30,000 cells per well into a white clear-bottom 96-well plate in 100 µl of growth medium. Incubate cells at 37°C in a CO_2 incubator overnight.

2. Next day, transfect 1 µl of SBE luciferase reporter (component A) into cells following the procedure in **Generalized Transfection and Assay Protocols.**

3. After ~24 hours of transfection, change media to 50 µl assay medium. Incubate cells at 37°C in a CO_2 incubator for ~ 4-5 hours.

4. After ~29 hours of transfection, set up each assay below in at least triplicate.
   Add three-fold serial dilution of human TGFβ1 in 5 µl of assay medium to stimulated wells.
   Add 5 µl of assay medium to unstimulated control wells.
   Add 55 µl of assay medium to cell-free control wells (for determining background luminescence).

5. Incubate at 37°C in a CO_2 incubator overnight (~18 hours).

6. Perform dual luciferase assay using BPS Dual Luciferase (Firefly-Renilla) Assay System (BPS Cat. #60683): Dilute 100x Firefly Luciferase Reagent Substrate (Component B) into Firefly Luciferase Reagent Buffer (Component A). Add 50 µl of Firefly Luciferase reagent per well and rock at room temperature for ~15 minutes, then measure firefly luminescence using a luminometer. Dilute 100x Renilla Luciferase Reagent Substrate (Component D) into Renilla Luciferase Reagent Buffer (Component C). Add 50 µl of Renilla Luciferase reagent per well, rock at room temperature for ~1 minute and measure Renilla luminescence.

7. To obtain the normalized luciferase activity for the SBE reporter, subtract background luminescence, then calculate the ratio of firefly luminescence from the SBE reporter to *Renilla* luminescence from the control *Renilla* luciferase vector.

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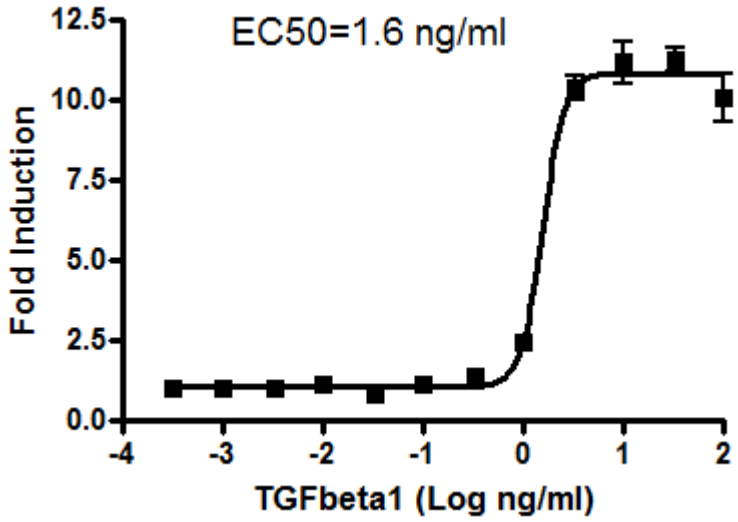
Figure 1. Dose response of SBE reporter activity to human TGFβ1. The results are shown as fold induction of normalized SBE luciferase reporter activity. Fold induction was determined by comparing values against the mean value for control cells without TGFβ1 treatment.

The EC50 of TGFβ1 is ~1.6 ng/ml.



Sample protocol to determine the effect of antagonists of TGFβ1/ SMAD signaling pathway on TGFβ1-induced SBE reporter activity in HEK293 cells

Additional materials required for this experiment

- SB525334 (Selleckchem #356559-20-1): inhibitor of TGFβ pathway. Make stock solution in DMSO.
- Human TGFβ1 (BPS Bioscience #90900-1)
- HEK293 growth medium: MEM/EBSS (with L-glutamine) (Hyclone #SH30024.01) + 10% FBS, 1% non-essential amino acids, 1 mM Na pyruvate + 1% Pen/Strep
- HEK293 assay medium: MEM/EBSS (with L-glutamine) (Hyclone #SH30024.01) + 0.5% FBS, 1% non-essential amino acids, 1 mM Na pyruvate + 1% Pen/Strep
- 96-well tissue culture-treated white clear-bottom assay plate (Corning # 3610)
- Dual Luciferase (Firefly-Renilla) Assay System (BPS Cat. #60683)

1. One day before transfection, seed HEK293 cells at a density of 30,000 cells per well into white clear-bottom 96-well plate in 100 µl of growth medium. Incubate cells at 37°C in a CO_2 incubator overnight.

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2. Next day, transfect 1 µl of SBE luciferase reporter (component A) into cells following the procedure in **Generalized Transfection and Assay Protocols.**

3. After ~24 hours of transfection, treat transfected cells with three-fold serial dilution of SB525334 in 50 µl assay medium. Incubate cells at 37°C in a CO_2 incubator for ~ 4-5 hours. For wells without SB525334, change to 50 µl assay medium with no treatment.

4. After ~29 hours of transfection, set up each assay below in at least triplicate.
   Add human TGFβ1 (final concentration 20 ng/ml) in 5 µl of growth medium to stimulated wells (cells treated with TGFβ1, with or without SB525334).
   Add 5 µl of assay medium to the unstimulated control wells (for determining the basal activity).
   Add 55 µl of growth medium to cell-free control wells (for determining background luminescence).

5. Incubate at 37°C in a CO_2 incubator for overnight (~18 hours).

6. Perform dual luciferase assay using BPS Dual Luciferase (Firefly-Renilla) Assay System (BPS Cat. #60683): Dilute 100x Firefly Luciferase Reagent Substrate (Component B) into Firefly Luciferase Reagent Buffer (Component A). Add 55 µl of Firefly Luciferase reagent per well and rock at room temperature for ~15 minutes, then measure firefly luminescence using a luminometer. Dilute 100x Renilla Luciferase Reagent Substrate (Component D) into Renilla Luciferase Reagent Buffer (Component C). Add 55 µl of Renilla Luciferase reagent per well, rock at room temperature for ~1 minute and measure Renilla luminescence.

7. To obtain the normalized luciferase activity of the SBE reporter, subtract background luminescence, then calculate the ratio of firefly luminescence from the SBE reporter to *Renilla* luminescence from the control *Renilla* luciferase vector.

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Figure 2. Inhibition of TGFβ1-induced SBE reporter activity by SB525334.

Figure 2a. SB525334 completely blocks TGFβ1-induced SBE reporter activity.

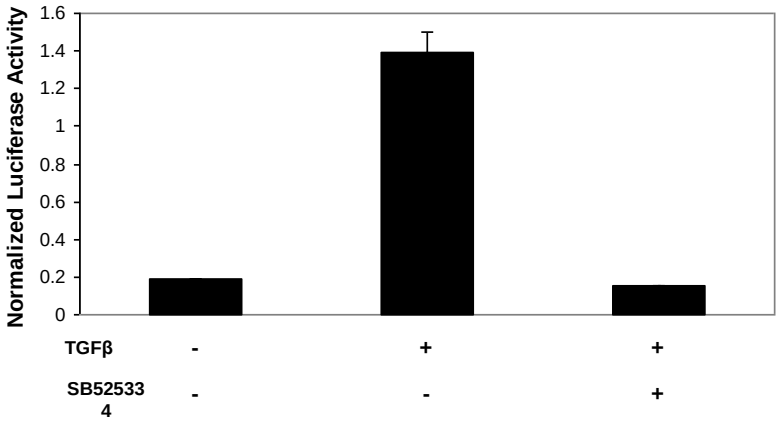
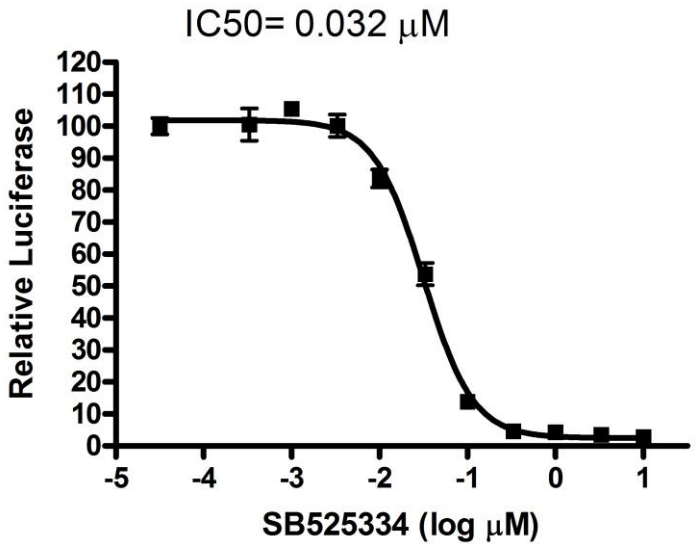


Figure 2b. Dose response of TGFβ1-induced SBE reporter activity to SB525334. The results are shown as percentage of SBE reporter activity. The normalized luciferase activity for cells stimulated with TGFβ1 in the absence of SB525334 was set at 100%.

The IC50 of SB525334 is ~ 0.032 μM.



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Reference

Moustakas A *et al.* (2001) Smad regulation in TGF-beta signal transduction. *J. Cell Science.* **114(Pt 24):** 4359-69.

Related Products

Product	Cat. #	Size
Dual Luciferase (Firefly-Renilla) Assay System	60683-1	10 mL
Dual Luciferase (Firefly-Renilla) Assay System	60683-2	100 mL
Dual Luciferase (Firefly-Renilla) Assay System	60683-3	1 L
SBE Reporter – HEK293 Cell Line	60653	2 vials
TGFβ1, Active Protein	90900-1	1 µg
TGFβ1, Active Protein	90900-2	5 µg
TGFβ1, Active Protein	90900-10	10 µg
TGFβ1, Active Protein	90900-3	1000 µg
TGFβ1, Latent Protein	90901-1	5 µg