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Data Sheet

The Transfection Collection™ – SBE Transient Pack
(TGFβ/SMAD Signaling Pathway)
Catalog #: 79272

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 Transient Pack is designed to provide the tools necessary for transiently transfecting and monitoring the activity of TGFβ/SMAD signaling pathway in the cultured cells. The kit contains transfection-ready vectors containing firefly luciferase as a TGFβ/SMAD pathway-responsive reporter and constitutively expressing Renilla luciferase as a transfection control. It also includes the TWO-Step Luciferase detection reagents to detect both luciferase activities and specialized medium for growing and assaying HEK293 cells.

The key to the SBE Transient Pack is the 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 pack 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.

Additionally, the pack includes cell culture medium (BPS Medium 1) that has been optimized for use with HEK293 cells*. BPS Medium 1 includes MEM medium, 10% fetal bovine serum, 1% non-essential amino acids, sodium pyruvate, and 1% Pen/Strep. Finally, the pack provides the TWO-Step Luciferase (Firefly & Renilla) Assay System. These luciferase reagents provide highly sensitive, stable detection of firefly luciferase activity and Renilla luciferase activity. The TWO-step luciferase reagents can be used directly in cells in growth medium, and can be detected with any luminometer; automated injectors are not required.

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*Note: the kit may be used with other cell lines than HEK293, but an alternate cell culture medium may be required for optimal cell growth.

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.

Components

Component	Amount	Storage
Reporter (Component A) SBE luciferase reporter vector* + constitutively expressing Renilla luciferase vector*	500 µl (60 ng DNA/ µl)	-20°C
Negative Control Reporter (Component B) Non-inducible luciferase vector*+ constitutively expressing Renilla luciferase vector*	500 µl (60 ng DNA/ µl)	-20°C
Firefly Luciferase Reagent Buffer	10 ml	-20°C
Firefly Luciferase Reagent Substrate (100x)	100 µl	-20°C *Protect from light*
Renilla Luciferase Reagent Buffer	10 ml	Room Temp.
Renilla Luciferase Reagent substrate (100x)	100 µl	-20°C *Protect from light*
BPS Medium 1	100 ml	+4°C

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

Materials Required but Not Supplied

- HEK293 cells. Other mammalian cell lines, such as HeLa, can be used, but an alternate cell culture medium may be required for optimal cell growth
- 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)
- Luminometer

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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 provided on a per well basis.

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

2. The 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 a "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.

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3. Carefully remove and discard 30 µl of media from each of the wells of cell culture, taking care not to disturb the cells or touch the bottom of the well with the pipet tip. Add the 30 µl of the complexes to each well containing 70 µl 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 TWO-Step Luciferase Assay System (below).

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

TWO-Step Luciferase Assay Procedure

1. Thaw **Firefly Luciferase Reagent Buffer** by placing the reagent in a room temperature water bath. Equilibrate the buffer to room temperature and mix well before use. *Note: It is important that the **Firefly Luciferase Reagent Buffer** be at room temperature before use.*
2. Calculate the amount of Firefly Luciferase Assay Working solution needed for the experiment (**Firefly Luciferase Reagent Buffer** + **Firefly Luciferase Reagent Substrate**). Immediately prior to performing the experiment, prepare the Firefly Luciferase Assay Working Solution by diluting **Firefly Luciferase Reagent Substrate** into **Firefly Luciferase Reagent Buffer** at a 1:100 ratio and mix well. Avoid exposing to excessive light. *Only use enough of each component for the experiment, remaining* **Firefly Luciferase Reagent Buffer** *and* **Firefly Luciferase Reagent Substrate** *should be stored separately at* -20°C.
3. Remove multi-well plate containing mammalian cells from incubator. *Note: plates must be compatible with luminescence measurement with luminometer being used.*
4. Add equal volume of Firefly Luciferase Assay Working Solution (step 2) to the culture medium in each well. Example: 96-well plate with 100 µl of culture medium requires 100 µl of Firefly Luciferase Assay Working Solution per well.

   Gently rock the plates for ≥15 minutes at room temperature. Measure firefly luminescence using a luminometer. The signal under these conditions will be stable for more than 2 hours at room temperature. For maximal light intensity, measure samples within 1 hour of reagent addition.
5. Calculate the amount of Renilla Luciferase Assay Working Solution needed for the experiment (**Renilla Luciferase Reagent Buffer** + **Renilla Luciferase Reagent Substrate**). Prepare the Renilla Luciferase Assay Working Solution by diluting **Renilla Luciferase Reagent Substrate** into **Renilla Luciferase Reagent Buffer** at a 1:100 ratio and mix well. Avoid exposing to excessive heat or light. *Only use enough of each component for the experiment,*

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6. Add equal volume of Renilla Luciferase Assay Working Solution (step 5) to each well. Example: 96-well plate with 100 µl of culture medium + 100 µl Firefly Luciferase Reagent requires 100 µl of Renilla Luciferase Assay Working Solution per well.
7. Gently rock the plates for ~1 minute at room temperature. Measure Renilla luminescence using a luminometer.
8. Data analysis: subtract background (wells with medium and luciferase reagent only) from all the readings. To obtain the normalized luciferase activity for the SBE reporter, subtract the background luminescence, then calculate the ratio of firefly luminescence from SBE reporter to Renilla luminescence from the control Renilla luciferase vector. To determine the fold induction, divide the value for the treated cells by the value for the untreated cells.

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)
- Opti-MEM I Reduced Serum Medium
- Opti-MEM I Reduced Serum Medium + 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)

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 BPS Medium 1. 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 Opti-MEM I Reduced Serum Medium + 0.5% FBS, 1% non-essential amino acids, 1 mM Na pyruvate + 1% Pen/Strep. 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 Opti-MEM I Medium to stimulated wells.
   Add 5 µl of Opti-MEM I Medium medium to unstimulated control wells.
   Add 55 µl of Opti-MEM I Medium to cell-free control wells (for determining background luminescence).

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

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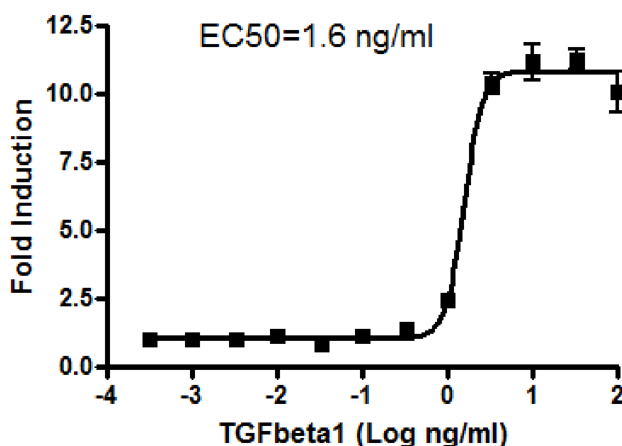
6. Perform TWO-step luciferase assay as described above in TWO-Step Luciferase Assay Procedure. Briefly, dilute 100x Firefly Luciferase Reagent Substrate into Firefly Luciferase Reagent Buffer that has been equilibrated to room temperature. Add 50 µl of Firefly Luciferase reagent per well and rock at room temperature for ~15 minutes, then measure firefly luminescence using a luminometer. Dilute 100x Renilla Luciferase Reagent Substrate into Renilla Luciferase Reagent Buffer. 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.

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 (Selleck, #356559-20-1): inhibitor of TGFβ pathway. Make stock solution in DMSO.
- Human TGFβ1 (BPS Bioscience, #90900-1)
- Opti-MEM I Reduced Serum Medium
- 96-well tissue culture-treated white clear-bottom assay plate (Corning, #3610)
- Opti-MEM I Reduced Serum Medium + 0.5% FBS, 1% non-essential amino acids, 1 mM Na pyruvate + 1% Pen/Strep

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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 BPS Medium 1. 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, treat transfected cells with three-fold serial dilution of SB525334 in 50 µl Opti-MEM I Reduced Serum Medium + 0.5% FBS, 1% non-essential amino acids, 1 mM Na pyruvate + 1% Pen/Strep 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 assay medium (Opti-MEM I Reduced Serum Medium + 0.5% FBS, 1% non-essential amino acids, 1 mM Na pyruvate + 1% Pen/Strep) 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 assay 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 TWO-step luciferase assay as described above in **TWO-Step Luciferase Assay Procedure**. Briefly, dilute 100x Firefly Luciferase Reagent Substrate into Firefly Luciferase Reagent Buffer that has been equilibrated to room temperature. 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 into Renilla Luciferase Reagent Buffer. 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.

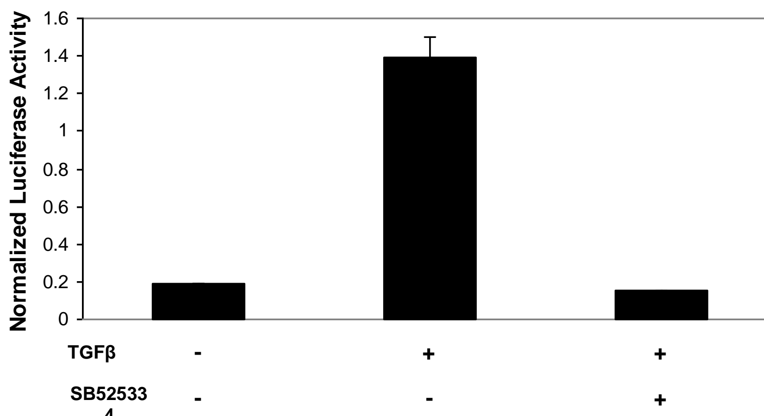
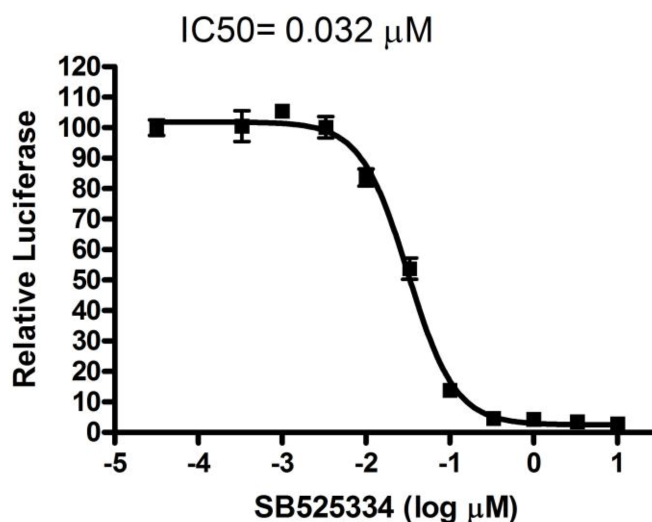


Figure 2b. Dose response of TGFβ1-induced SBE reporter activity to SB525334. The results are shown as percentage of SBE reporter activity. The normalized luciferase activity for cells stimulated with TGFβ1 in the absence of SB525334 was set at 100%.

The IC50 of SB525334 is ~ 0.032 μM.



Reference

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

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Refills

Product	Cat. #	Size
TWO-Step Luciferase (Firefly & Renilla) Assay System	60683-1	10 mL
TWO-Step Luciferase (Firefly & Renilla) Assay System	60683-2	100 mL
TWO-Step Luciferase (Firefly & Renilla) Assay System	60683-3	1 L
SBE Reporter Kit	60654	500 rxns.
BPS Medium 1	79259	100 ml

Related Products

Product	Cat. #	Size
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

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