

Data Sheet

FOXO Reporter Kit (PI3K/AKT Pathway) Catalog. #w70654

Background

The PI3K/AKT signaling pathway is essential for cell growth and survival. Disruption of this pathway or its regulation has been linked to a variety of cancers and coronary diseases. Mammalian FOXO protein (FOXO1, FOXO3, FOXO4), a subgroup of Forkhead transcription factors, is among the best characterized targets of the PI3K/AKT signaling pathway. These transcription factors function as a trigger for apoptosis by up-regulating genes necessary for cell death. Insulin or growth factors induce activation of PI3K, which in turn activates AKT. AKT directly phosphorylates FOXOs, resulting in the export of FOXOs from the nucleus to the cytoplasm, thereby inhibiting FOXO-dependent transcription.

Description

The FOXO Reporter kit is designed for monitoring the activity of the PI3K/AKT signaling pathway and the transcriptional activity of FOXO proteins in cultured cells. The kit contains the transfection-ready FOXO3 expression vector and the FOXO luciferase reporter vector, which is a PI3K/Akt pathway-responsive reporter. This reporter contains the firefly luciferase gene under the control of multimers of the FOXO responsive element located upstream of a minimal promoter. The FOXO reporter is premixed with a constitutively-expressing sea pansy (*Renilla*) luciferase vector, which serves as an internal control for the 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 also contains the firefly luciferase gene under the control of a minimal promoter, but without any additional response elements. The negative control is critical to determining pathway-specific effects and background luciferase activity.

Application

- Monitor PI3K/Akt signaling pathway activity and FOXO transcriptional activity.
- Screen for activators or inhibitors of the PI3K/AKT signaling pathway.
- Study effects of RNAi or gene overexpression on the activity of the PI3K/AKT pathway.

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Components

Component	Specification	Amount	Storage
Reporter (Component A)	FOXO luciferase reporter vector + constitutively-expressing <i>Renilla</i> luciferase vector	500 µl (60 ng DNA/ µl)	-20°C
Negative Control Reporter (Component B)	Non-inducible luciferase vector + constitutively-expressing <i>Renilla</i> luciferase vector	500 µl (60 ng DNA/ µl)	-20°C
FOXO3 (Component C)	Expression vector for FOXO3	250 µl (100 ng DNA/µl)	-20°C
Negative Control Expression vector (Component D)	Empty expression vector without FOXO3	250 µl (100 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 line [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-Glo® Luciferase Assay System (Promega #E2920): This system assays cells directly in growth medium. It can be used with any luminometer. Automated injectors are not required.
 - OR
 - Dual-Luciferase® Reporter Assay System (Promega #E1910): This system requires a cell lysis step. It is ideal for luminometers with automated injectors.
- 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 given on a per well basis.

1. One day before transfection, seed cells at a density of ~ 30,000 cells per well in 100 μ l of growth medium so that cells will be 90% confluent at the time of transfection.
2. The next day, for each well, prepare complexes as follows:
 - a. Dilute DNA mixtures in 15 μ l of Opti-MEM I medium (antibiotic-free). Mix gently.

Depending upon the experimental design, the DNA mixtures may be any of following combinations:

- **1 μ l of Reporter** (component A); in this experiment, the control transfection is **1 μ l of Negative Control Reporter** (component B).
- **1 μ l of Reporter** (component A) + **experimental vector** expressing gene of interest (such as component C); in this experiment, the control transfection is: **1 μ l of Reporter** (component A) + **negative control expression vector** (such as component D), **1 μ l of Negative Control Reporter** (component B) + **experimental vector** expressing gene of interest (component C), and **1 μ l of Negative Control Reporter** (component B) + **negative control expression vector** (component D).
- **1 μ l of Reporter** (component A) + specific siRNA; in this experiment, the control transfection is: **1 μ l of Reporter** (component A) + negative control siRNA, **1 μ l of Negative Control Reporter** (component B) + specific siRNA, and **1 μ l of Negative Control Reporter** (component B) + negative control siRNA.

Note: we recommend setting up at least triplicate assays for each condition, and preparing transfection cocktails for multiple wells to minimize pipetting errors.

- b. Mix Lipofectamine 2000 gently before use, then dilute 0.35 μ l of Lipofectamine 2000 in 15 μ l 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. Add the 30 μ l of complexes to each well containing cells and medium. Mix gently by tapping the plate.
4. Incubate cells at 37°C in a CO₂ incubator. 24 to 48 hours after transfection, perform dual luciferase assay following the manufacturer's protocol.

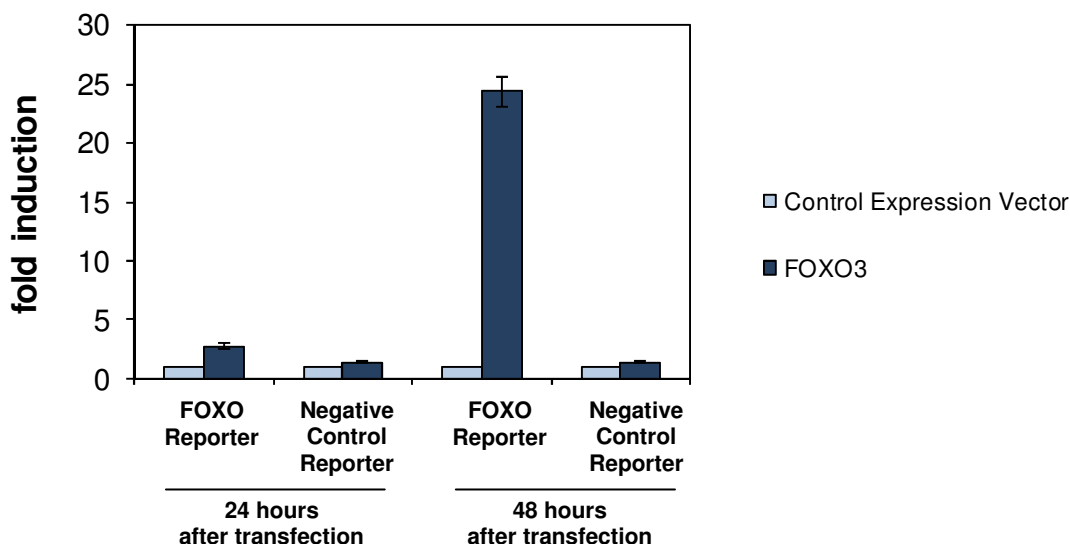
To study the effect of activators / inhibitors on the PI3K/AKT pathway, treat cells with test compound 6 hours or 24 hours after transfection. Perform dual luciferase assay 24-48 hours after transfection.

Sample protocol to determine the effect of FOXO3 on FOXO reporter activity in HEK293 cells

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 μ l of growth medium (MEM/EBSS (Hyclone #SH30024.01), 10% FBS, 1% non-essential amino acids, 1mM Na-pyruvate, 1% Pen/Strep). Incubate cells overnight at 37°C in a CO₂ incubator.
2. The next day, transfect 1 μ l of FOXO reporter (component A) with 0.5 μ l of FOXO3 (component C) or negative control expression vector (component D) into cells following the procedure in **Generalized Transfection and Assay Protocols**.
3. After ~24 hours or ~48 hours of transfection, perform the dual luciferase assay using Dual-Glo[®] Luciferase Assay System (Promega #E2920): remove 80 μ l of medium from each well. Add 50 μ l of growth medium to cell-free control wells (for determining background luminescence). Add 50 μ l of Luciferase reagent per well and rock at room temperature for ~15 minutes, then measure the firefly luminescence using a luminometer. Add 50 μ l of Stop & Glo reagent per well and rock at room temperature for ~15 minutes, then measure the *Renilla* luminescence.
4. To obtain the normalized luciferase activity for the FOXO reporter, subtract the background luminescence, then calculate the ratio of firefly luminescence from FOXO reporter to *Renilla* luminescence from the control *Renilla* luciferase vector.

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Figure 1. FOXO3 induced expression of the FOXO reporter. The results are shown as fold induction of normalized reporter activity by FOXO3 (ratio of normalized reporter activity in the presence of FOXO3 to that in the presence of the negative control expression vector).



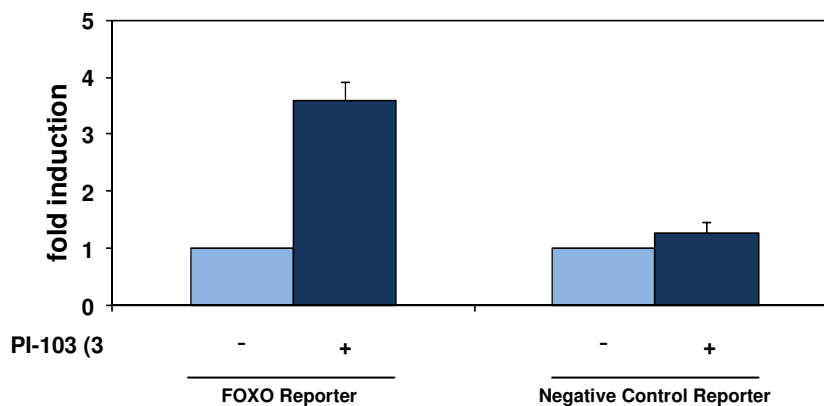
Sample protocol to determine the effect of PI3K inhibitors on FOXO reporter activity in HEK293 cells

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 μ l of growth medium. Incubate cells overnight at 37°C in a CO₂ incubator.
2. The next day, transfect 1 μ l of FOXO-Luc luciferase reporter (component A) with 0.5 μ l of FOXO3 (component C) or negative control expression vector (component D) into cells following the procedure in **Generalized Transfection and Assay Protocols**.
3. After ~6 hours of transfection, change medium to 50 μ l of fresh growth medium with or without the PI3K inhibitor, PI-103. Add 50 μ l of growth medium to cell-free control wells (to determine background luminescence).
4. After ~24 hours of treatment, perform the dual luciferase assay using Dual-Glo® Luciferase Assay System (Promega #E2920): Add 50 μ l of Luciferase reagent per well. Rock at room temperature for ~15 minutes, then measure the firefly luminescence using a luminometer. Add 50 μ l of Stop & Glo reagent per well. Rock at room temperature for ~15 minutes, then measure the *Renilla* luminescence.

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

Figure 2. The PI3K inhibitor, PI-103, up-regulates FOXO3-induced FOXO reporter activity. The results are shown as fold induction of FOXO3-induced reporter activity by PI-103.



References

- Essaghiri, A., *et al.* (2009) The transcription of FOXO genes is stimulated by FOXO3 and repressed by growth factors. *J. Biol. Chem.* **284**(16):10334-10342
- Hennessey B.T., *et al.* (2005) Exploiting the PI3K/AKT pathway for cancer drug discovery. *Nat. Rev. Drug Discov.* **4**(12):988-1004.

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