Data Sheet

FOXO Reporter Kit (PI3K/AKT Pathway)
Catalog. #AMS.60643

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 *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
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 Luciferase (Firefly-Renilla) Assay System (Cat.#AMS.60683): This system assays cells directly in growth medium. It can be used with any luminometer. Automated injectors are not required.
- 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 µl 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 µl of Opti-MEM I medium (antibiotic-free). Mix gently.

   Depending upon the experimental design, the DNA mixtures may be any of 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 (such as component C); in this experiment, the control transfection is: **1 µl of Reporte**r (component A) + **negative control expression vector** (such as component D), **1 µl of Negative Control Reporter** (component B) + **experimental vector** expressing gene of interest (component C), and **1 µl of Negative Control Reporter** (component B) + **negative control expression vector** (component D).
   - **1 µl of Reporter** (component A) + specific siRNA; in this experiment, the control transfection is: **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 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 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. 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. 24 to 48 hours after transfection, perform the Dual Luciferase Assay System following the protocol on the data sheet (Cat. #AMS.60683).

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 µl 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 CO_2 incubator.

2. The next day, transfect 1 µl of FOXO reporter (component A) with 0.5 µl 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 dual luciferase assay using Dual Luciferase (Firefly-Renilla) Assay System (Cat #AMS.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.

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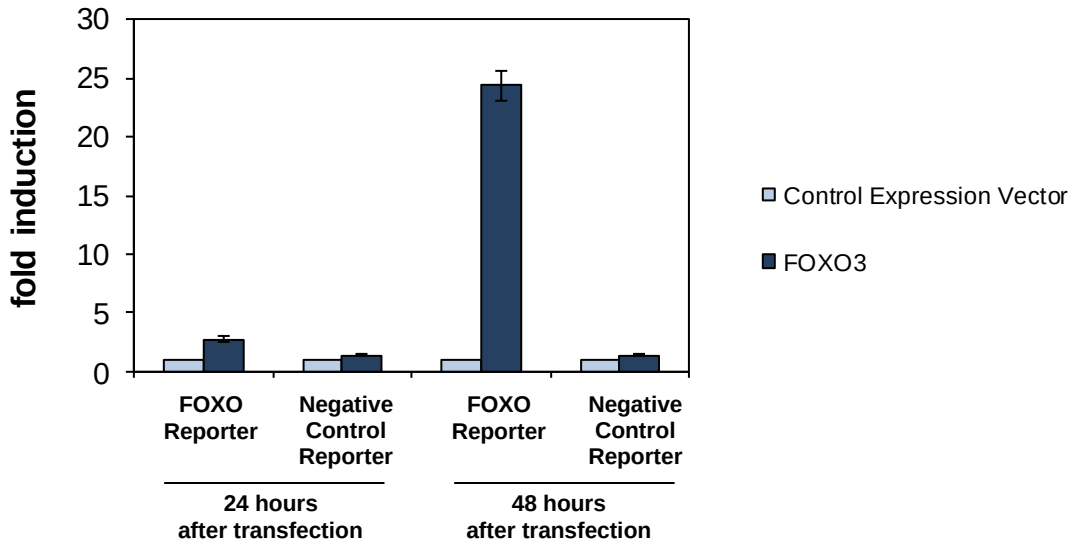
Figure 1. FOXO3 induced expression of the FOXO reporter. The results are shown as fold induction of normalized reporter activity by FOXO3 (ratio of normalized reporter activity in the presence of FOXO3 to that in the presence of the negative control expression vector).



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 µl of growth medium Incubate cells overnight at 37°C in a CO_2 incubator.

2. The next day, transfect 1 µl of FOXO-Luc luciferase reporter (component A) with 0.5 µl 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 µl of fresh growth medium with or without the PI3K inhibitor, PI-103. The final concentration of PI-103 should be 3 µM in the wells. Add 50 µl of growth medium to cell-free control wells (to determine background luminescence).

4. After ~24 hours of treatment, perform dual luciferase assay using Dual Luciferase (Firefly-Renilla) Assay System (Cat. AMS.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

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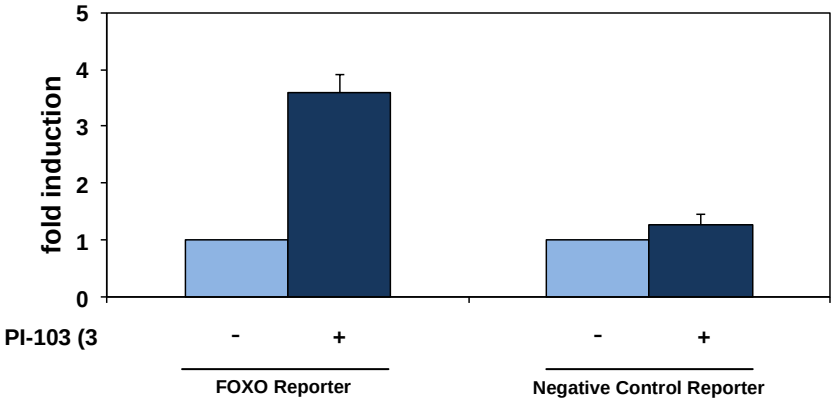
measure firefly luminescence using a luminometer. Dilute 100x Renilla Luciferase Reagent Substrate (Component D) into Renilla Luciferase Reagent Buffer (Component C). Add 50 µl of Renilla Luciferase reagent per well, rock at room temperature for ~1 minute and measure Renilla luminescence.

5. 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

Essaghir, 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

Hennessy B.T., *et al*. (2005) Exploiting the PI3K/AKT pathway for cancer drug discovery. *Nat. Rev. Drug Discov.* **4(12):**988-1004.

Related Products:

AKT1 Active Enzyme	Cat. AMS.40003	10 µg
AKT2 Active Enzyme	Cat. AMS.40011	10 µg
AKT3 Active Enzyme	Cat. AMS.40012	10 µg
Dual Luciferase (Firefly-Renilla) Assay System	Cat. AMS.60683-1	10 mL
Dual Luciferase (Firefly-Renilla) Assay System	Cat. AMS.60683-2	100 mL
Dual Luciferase (Firefly-Renilla) Assay System	Cat. AMS.60683-3	1 L
PI3 kinase (p110α/p85α)	Cat. AMS.40620	20 µg

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PI3 kinase (p110β/p85α)	Cat. AMS.40622	20 µg
PI3 kinase (p110δ/p85α)	Cat. AMS.40628	20 µg
PI3 kinase (p120γ)	Cat. AMS.40620	20 µg
CRE/CREB Reporter Assay Kit	Cat. AMS.60611	500 rxns.
TCF/LEF Reporter Kit (WNT)	Cat. AMS.60500	500 rxns.
Notch pathway Reporter Kit	Cat. AMS.60509	500 rxns.

AMSBIO| www.amsbio.com | info@amsbio.com

UK & Rest of the World
184 Park Drive, Milton Park
Abingdon OX14 4SE, UK
T: +44 (0)1235 828 200
F: +44 (0) 1235 820 482

North America
1035 Cambridge Street,
Cambridge, MA 02141
T: +1 (617) 945-5033 or
T: +1 (800) 987-0985
F: +1 (617) 945-8218

Germany
Bockenheimer Landstr. 17/19
60325 Frankfurt/Main
T: +49 (0) 69 779099
F: +49 (0) 69 13376880

Switzerland
Centro Nord-Sud 2E
CH-6934 Bioggio-Lugano
T: +41(0) 91 604 55 22
F: +41(0) 91 605 17 85