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Data Sheet

ARE Reporter Kit Nrf2 *(*Antioxidant Pathway)
Catalog #: w70525

Background

The Nrf2 antioxidant response pathway plays an important role in the cellular antioxidant defense. Nrf2, a basic leucine zipper transcription factor, induces the expression of antioxidant and phase II enzymes by binding to the ARE (antioxidant response element) region of the gene promoter. Under basal conditions, Nrf2 is retained in the cytosol by binding to the cytoskeletal protein Keap1. Upon exposure to oxidative stress and other ARE activators, Nrf2 is released from Keap1 and translocates to nucleus, where it can bind to the ARE, leading to the expression of antioxidant and phase II enzymes that protect the cell from oxidative damage.

Description

The ARE Reporter kit is designed for monitoring the activity of the Nrf2 antioxidant pathway in cultured cells. The kit contains a transfection-ready ARE luciferase reporter vector, which is an Nrf2 pathway-responsive reporter. This reporter contains a firefly luciferase gene under the control of multimerized ARE responsive elements located upstream of a minimal promoter. The ARE reporter is premixed with a constitutively-expressing *Renilla* luciferase vector that serves as an 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. This negative control is critical to determining pathway-specific effects and background luciferase activity.

Application

- Monitor Nrf2 antioxidant response pathway activity.
- Screen for activators or inhibitors of the Nrf2 antioxidant response pathway.
- Study effects of RNAi or gene overexpression on the activity of the Nrf2 pathway.

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Components

Component	Specification	Amount	Storage
Reporter (Component A)	ARE 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

Note: 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
- Transfection reagent for mammalian cell line [We use Lipofectamine™ 2000 (Life technologies # 11668027). However, other transfection reagents work equally well.]
- Opti-MEM I Reduced Serum Medium (Life technologies #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

Generalized Transfection and Assay Protocols

The following procedure is designed to transfect the reporter to HepG2 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 ~ 35,000 cells per well in 100 µl of growth medium.

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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 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 transfection is: **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 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 each condition in at least triplicate, and preparing 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.

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

7. Incubate cells at 37°C in a CO_2 incubator overnight.

8. The next day, induce the ARE reporter with medium containing activators of the Nrf2 pathway. Incubate cells at 37°C in a CO_2 incubator for ~ 16 to 24 hours, then perform the dual luciferase assay following the manufacturer's protocol.

Sample protocol to determine the effect of antioxidant inducers on ARE reporter activity in HepG2 cells

1. One day before transfection, seed HepG2 cells at a density of 35,000 cells per well into white clear-bottom 96-well plate in 100 µl of growth medium (MEM/EBSS (Hyclone

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#SH30024.01), 10% FBS, 1% non-essential amino acids, 1 mM Na-pyruvate, 1% Pen/Strep). Incubate cells overnight at 37°C in a CO_2 incubator.

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

3. Incubate cells at 37° in a CO_2 incubator overnight.

4. The next day after transfection, dilute antioxidant inducer, DL-Sulforaphane, in growth medium to 10 µM. Remove medium from cells and add 50 µl of diluted DL-Sulforaphane to wells. The final DMSO concentration is 0.1%.

   Add 50 µl of growth medium with 0.1% of DMSO to the unstimulated control wells.
   Add 50 µl of growth medium with 0.1% of DMSO to cell-free control wells (to determine background luminescence).

   Set up each treatment in at least triplicate.

5. Incubate cells at 37°C in a CO_2 incubator for ~ 16 to 24 hours.

6. Perform dual luciferase assay using Dual-Glo® Luciferase Assay System (Promega #E2920): Add 50 µl of Luciferase reagent per well and rocking at room temperature for ~15 minutes, then measure firefly luminescence using a luminometer. Add 50 µl of Stop & Glo reagent per well. Rock at room temperature for ~15 minutes and measure Renilla luminescence.

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

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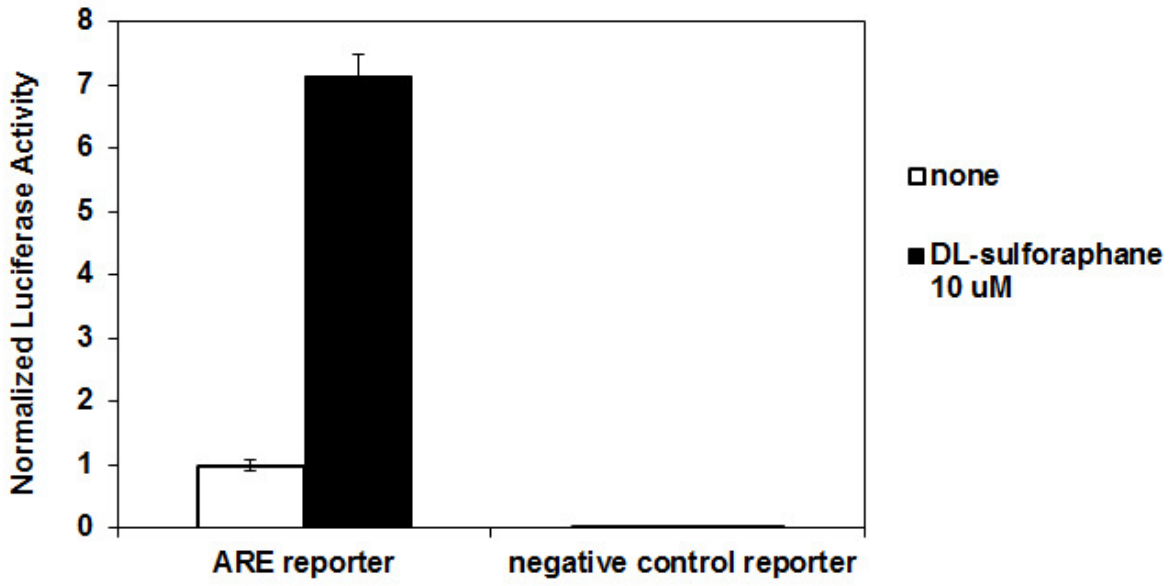
Figure 1. DL-Sulforaphane, an antioxidant inducer, induces the expression of ARE reporter in HepG2 cells. The results are shown as normalized ARE luciferase reporter activity.



References

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Dinkova-Kostova AT *et.al.* (2002) Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proc. Natl. Acad. Sci. USA.* **99(18):** 11908-11913.