Description

The Keap1:Nrf2 Inhibitor Screening Assay Kit is designed for identification of inhibitors of Keap1:Nrf2 binding using fluorescence polarization (FP), a powerful tool for studying molecular interactions by monitoring the change in rotational mobility of the fluorescently-labeled molecules upon their binding to a partner. The 96-well format assay kit contains sufficient amounts of purified Keap1 protein, fluorochrome-conjugated Nrf2 peptide containing the ETGE motif, and assay buffer for 100 binding reactions. To determine the effect of the inhibitor on the formation of Keap1:Nrf2 complexes the Keap1 protein and the fluorescent Nrf2 peptide are incubated with or without the test inhibitor for 30 minutes. Changes in rotational mobility of the Nrf2 peptide are measured using a plate reader *capable of measuring fluorescence polarization.*

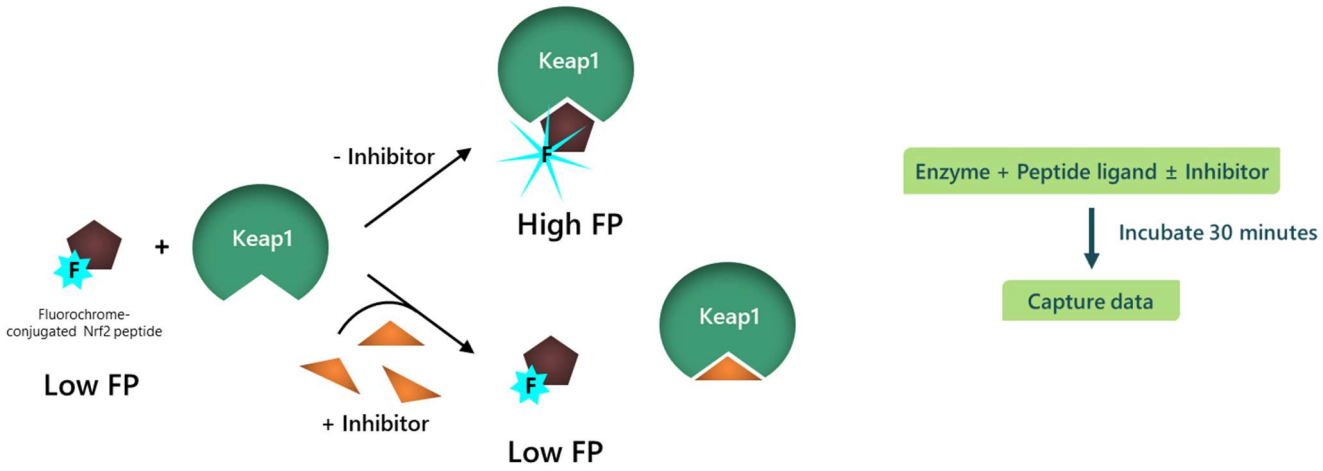

Figure 1. Illustration of the Keap1:Nrf2 Inhibitor Screening Assay Kit.

Background:

Nuclear factor erythroid 2-related factor (Nrf2) is a transcription factor that coordinates antioxidant and anti-inflammatory responses by increasing the expression of antioxidant proteins through binding to the *ARE* (antioxidant response element) region of their promoters. Under basal conditions, Nrf2 is retained in the cytosol by cytoskeletal Keap1, a substrate recognition subunit of a E3 ligase complex which targets Nrf2 for degradation. Exposure to oxidative stress causes the release of Nrf2 from Keap1. Nrf2 translocates to the nucleus, where it binds to *AREs* and induce the expression of antioxidant and phase II proteins protecting the cell from oxidative damage. Both Nrf2 and Keap1 are attractive therapeutic targets.

Applications

Great for studying enzyme kinetics and screening small molecule inhibitors for drug discovery and high-throughput applications.

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Supplied Materials

Catalog #	Name	Amount	Storage	
70040	Keap1, Human Recombinant*	30 µg	-80°C	***Avoid multiple freeze/ thaw cycles***
79112	FAM-Nrf2 peptide (10 µM)	10 µl	-80°C	
79113	Keap1-Nrf2 Assay Buffer	10 ml	-20°C	
	BSA, 10 mg/ml	100 µl	-80°C	
79685	96-well black plate	1	Room Temp	

**The concentration of the protein is lot-specific and will be indicated on the tube*

Materials Required but Not Supplied

Adjustable micropipettor and sterile tips

Plate reader capable of fluorescence polarization measurements, λex = 475-495 nm, λem = 518-538 nm.

Storage Conditions

This assay kit will perform optimally for up to 6 months from date of receipt when the materials are stored as directed. ***Avoid multiple freeze/ thaw cycles!***

Safety

This product is for research purposes only and not for human or therapeutic use. This product should be considered hazardous and is harmful by inhalation, in contact with skin, eyes, clothing, and if swallowed. If contact occurs, wash thoroughly.

Assay Protocol

- All samples and controls should be performed in triplicates.
- The assay should include a "Blank", a "Negative control" and a "Positive control" in addition to the "Test Inhibitor".

1. Thaw fluorescent Nrf2 peptide on ice. Briefly spin the tube containing the protein to recover its full contents. If the assay plate is going to be used more than once, prepare enough peptide for this portion of the assay and aliquot the remaining undiluted peptide into single-use aliquots depending on how many times the assay plate will be used. Store the aliquots at -80°C.

2. Dilute the fluorescent Nrf2 peptide 1:10 in Assay Buffer. Keep the diluted peptide on ice until use.

3. Prepare the Master Mix: N wells × (24 µl of Assay Buffer + 0.5 µl of BSA (10 mg/ml) + 0.5 µl of diluted Nrf2 peptide).

4. Add 25 µl of Master Mix to each well labeled "Test Inhibitor, "Positive Control", and "Negative Control". For the "Blank", add 45 µl of Assay Buffer.

5. Prepare the Test Inhibitor (5 µl/well, the final volume of the reaction is 50 µl).
   5.1. If the Test Inhibitor is water-soluble, prepare serial dilutions in the Assay Buffer at concentrations 10-fold higher than the desired final concentrations.

OR

5.2. If the Test inhibitor is soluble in DMSO, prepare the test inhibitor in 100% DMSO at a concentration 100-fold higher than the highest desired final concentration, then dilute the inhibitor 10-fold in Assay Buffer to prepare the highest concentration of the serial dilutions. The concentration of DMSO is now 10%.
Using the Assay Buffer containing 10% DMSO to keep the concentration of DMSO constant, prepare serial dilutions of the Test Inhibitor at 10-fold the desired final concentrations.
For positive and negative controls, prepare 10% DMSO in Assay Buffer (vol/vol) so that all wells contain the same amount of DMSO (Diluent Solution).
Note: The final concentration of DMSO in the assay should not exceed 1%.

6. Add 5 µl of Test Inhibitor to wells labeled "Test Inhibitor."

7. For the "Positive Control", the "Negative control", and the "Blank," add 5 µl of the Diluent Solution (assay buffer, or assay buffer containing 10% DMSO).

8. Thaw **Keap1** protein on ice. Briefly spin the tube containing the protein to recover its full contents. If the assay plate is going to be used more than once, prepare enough protein for this portion of the assay and aliquot the remaining undiluted protein into single-use aliquots depending on how many times the assay plate will be used. Store the aliquots at -80°C.

   Note: Keap1 is very sensitive to freeze/thaw cycles. Do not re-use thawed aliquots more than once and do not reuse the diluted protein.

9. Dilute **Keap1** in Assay buffer to 15 ng/µl (300 ng/reaction). Keep the protein on ice until use.

10. Add 20 µl of Assay Buffer to the "Negative Control".

11. Initiate the reaction by adding 20 µl of the diluted Keap1 protein to the "Positive control" and the "Test Inhibitor". Incubate at room temperature for 30 minutes.

12. Read the fluorescent polarization of the sample in a microtiter-plate reader capable of excitation at wavelengths ranging from 475-495 nm and detection of emitted light ranging from 518-538 nm. Blank value is subtracted from all other values.

	Blank	**Negative Control**	**Positive Control**	**Test Inhibitor**
Master Mix	-	25 µl	25 µl	25 µl
Assay Buffer	45 µl	20 µl	-	-
Diluent Solution	5 µl	5 µl	5 µl	-
Test Inhibitor	-	-	-	5 µl
Keap1 (15 ng/µl)	-	-	20 µl	20 µl
Total	**50 µl**	**50 µl**	**50 µl**	**50 µl**

Calculating Results:

Definition of Fluorescence Polarization

$$P = \frac{I_{II} - I_{\perp}}{I_{II} + I_{\perp}}$$

where $I_{//}$ is the Intensity with polarizers parallel and $I_{\perp}$ is the Intensity with polarizers perpendicular. Most instruments display fluorescence polarization in units of mP.

$$mP = \left(\frac{I_{II} - I_{\perp}}{I_{II} + I_{\perp}}\right) x\ 1000$$

The equation above assumes that light is transmitted equally well through both parallel and perpendicular oriented polarizers. In practice, this is generally not true and a correction must be made to measure the absolute polarization state of the molecule. This correction factor is called the "G Factor".

$$mP = \left(\frac{I_{II} - G(I_{\perp})}{I_{II} + G(I_{\perp})}\right) x\ 1000 \quad \text{or} \quad mP = \left(\frac{G(I_{II}) - I_{\perp}}{G(I_{II}) + I_{\perp}}\right) x\ 1000$$

The G-factor is instrument-dependent and may vary slightly depending upon instrument and conditions. Please check the manual of your instrument to obtain the information about the establishment of the G-factor.

Validation data

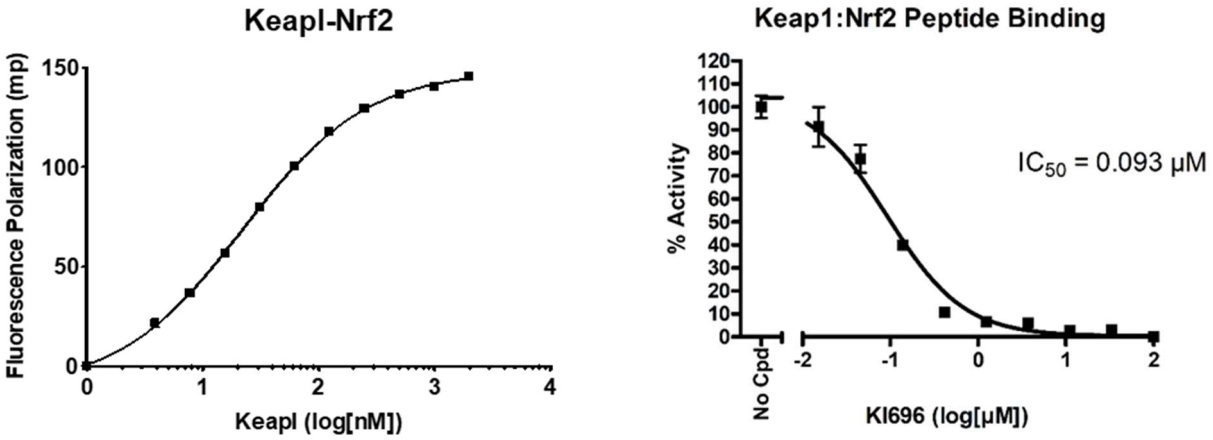

Figure 1: Left: Binding of Keap1 to Nrf2. Right: inhibition of Keap1:Nrf2 binding by Nrf2 inhibitor. Binding was measured using the Keap1:Nrf2 Inhibitor Screening Assay Kit, (BPS Bioscience #72020). Nrf2 inhibitor KI696 (Medchem Express #HY-101140) was incubated with Nrf2 peptide and Keap1 at increasing concentrations. Fluorescence polarization was measured at λex 485nm, λem 530 nm using a Bio-Tek fluorescent microplate reader.

Data shown is representative, for lot-specific information, please contact BPS Bioscience, Inc. at support@bpsbioscience.com.

Reference

Inoyama D. *et al*. (2012) *J. Biomol. Screening* **17(4):** 435-447.

Troubleshooting Guide

Visit bpsbioscience.com/assay-kits-faq for detailed troubleshooting instructions. For all further questions, please email support@bpsbioscience.com

Related Products

Products	*Catalog #*	*Size*
NRF2, FLAG-Tag Recombinant	101463	100 µg
KEAP1 (2-624), His-Tag Recombinant	101132	100 µg
ARE Reporter Kit (Nrf2 Antioxidant Pathway)	60514	500 reactions
ARE Luciferase Reporter HepG2 Cell Line (Nrf2 Antioxidant Pathway)	60513	2 vials
ARE Luciferase Reporter Lentivirus	79869	500 µl x 2