Northwest
Life Science Specialties, LLC

Premier Products for Superior Life Science Research

NWLSS™ Mouse KEAP1 ELISA

Product NWK-KEAP1M1

For Research Use Only

Simple assay kit for quantitative measurement of Mouse Kelch-like ECH-associated protein 1 (KEAP1)

Table of Contents

Introduction:

Nuclear factor, erythroid 2 like protein 2 (NFE2L2) commonly referred to as Nrf2 is an important protein in the regulation of oxidative and xenobiotic stress response. Kelch-like ECH-associated protein 1 (KEAP1), also known as inhibitor of Nrf2 (iNrf2), is equally important due to it being the primary regulator of Nrf2. In the cytosol, KEAP1 normally binds to Nrf2 preventing it from translocating to the nucleus and targeting it for ubiquitination and subsequent degradation by proteasomes. However, KEAP1 is a cysteine rich protein that is sensitive to oxidative stress. When exposed to the appropriate stressor, oxidation of C151, C273 and C288 can produce a conformation change in Keap1 causing release of Nrf2 which then allows translocation of Nrf2 to the nucleus. Inside the nucleus, Nrf2 is free to participate in Nrf2/sMAF/ARE transcription pathways and is capable of activating more than three dozen xenobiotic and oxidative stress response genes. Many studies have shown a positive correlation between active Nrf2 and disease amelioration whereas others have shown heightened NRF2 and/or lower KEAP1 levels to increase cancer cell viability and resistance to chemotherapy. In either role KEAP1 regulation of Nrf2 has emerged as a very important target for researchers trying to ascertain the full potential of the KEAP1/Nrf2/ARE pathway in terms of lessening the Deleterious effects of disease and aging.

Intended Use:

The NWLSS™ Mouse KEAP1 ELISA is intended for quantitative detection of Kelch-like ECH-associated protein 1 (KEAP1), also known as iNrf2.

Test Principle:

The NWLSS™ Mouse KEAP1 ELISA is a sandwich format Enzyme-Linked Immunosorbent Assay (ELISA). The assay features a microtiter plate pre-coated with a monoclonal antibody specific to mouse KEAP1. Briefly, Nrf2 in samples and standards is captured by the plate bound antibody and remaining liquid is removed. Next, a biotinylated antibody with specificity to KEAP1 is added and allowed to bind to KEAP1 captured in the previous step. After incubation, non-bound components are removed by washing. Avidin-HRP conjugate is then added and allowed to incubate. After a subsequent thorough wash TMB-substrate solution is added to each well which produces blue coloration wherever KEAP1 is present. Finally, a sulfuric acid stop solution is added and the resulting yellow colored product is measured at 450nm. The amount of KEAP1 in the sample can be easily determined by direct comparison of unknown sample absorbance with known standard curve absorbance values generated in the assay.

General Specifications:

Format::	1 X 96 wells	
Number of tests:	Triplicate =	24
	Duplicate =	40
Specificity:	Mouse KEAP1	
Sensitivity:	LLD = 60 pg/mL in sample applied to plate	
Standard Range:	0.156 ng/mL - 10 ng/mL	

Kit Contents:

Foil Pouch	Microtiter plate precoated with anti-Mouse KEAP1	12 X 8 wells
2 Vials	KEAP1 Standard (Purified Mouse KEAP1)	(Lyophilized)
1 Bottle	Standard Diluent	(20 mL)
1 Vial	100X Secondary Antibody	(120 µL)
1 Bottle	Secondary Antibody Diluent	(12 mL)
1 Vial	100X Avidin-HRP Conjugate	(120 µL)
1 Bottle	Avidin-HRP Diluent	(12 mL)
1 Bottle	TMB Substrate (3,3',5,5'tetramethylbenzidine)	(9 mL)
1 Bottle	Stop Solution: (1 N Sulfuric Acid, H_2SO_4)	(6 mL)
1 Bottle	30X Wash Buffer	(20 mL)
4	Adhesive Plate Covers	(Sheets)

Required Materials Not Provided:

Adjustable pipettes capable of transferring 50 µL to 200 µL volumes.
A multi-channel or repeater pipette (recommended).
Eppendorf tubes for sample & standard dilutions.
Reagent Trays (if using multi-channel).
37°C Incubator
Distilled water

Required Instrumentation:
Microplate reader with 450nm capability.

Warnings, Limitations, Precautions:
Stability Limitation:
The diluted Standards, Secondary Antibody and Detection Reagent should be used only once.

Standards:
Do not attempt to serially dilute the standards directly in the plate wells. Doing so will produce erroneous results. For best results, prepare the standards in separate tubes just prior to performing the assay.

Incubation Temperature
Measured values can be affected by variations in incubation temperatures. Exercise care to assure uniform temperature during each incubation. We recommend that incubation be performed in a humid environment such as in an incubator with water present.

TMB Substrate:
TMB Substrate is light sensitive: Keep it in the dark. Do not open until just before use.

Wash Solution:
If crystals have formed in the concentrated 30X Wash Solution, simply warm to room temperature and mix gently until the crystals are completely dissolved.

Blank wells:
Do not add Tracer Antibody to Blank wells.

Safety:
Individual components may be harmful if swallowed, inhaled or absorbed through the skin. Contact should be minimized through the use of gloves and standard good laboratory practices. If contact with skin or eyes occurs, rinse the site immediately with water and consult a physician.

Storage Instructions:
Upon receipt, the Standard, Tracer Antibody, Detection Reagent and the 96-well strip plate should be stored at -20 °C . The remaining components should be stored at 2-8°C.

This kit is stable if unopened for 6 months from the date of manufacture. Do not use components beyond the expiration date printed on the kit box label. Unused wells and opened reagents must be stored according to their recommended storage temperature and should be used within 30 days after first opening.

Assay Preparation

Plate Reader Setup

Wavelength:	450 nm
Mode:	Endpoint
Shaker:	On
Curve Fit:	2nd Order Polynomial

Reagent Preparation:

Bring all reagents, plate wells to be used samples and calibrators to room temperature (20-25°C) before use.

Wash Buffer:

Prepare the necessary volume of wash buffer by mixing 1 part **30X Wash Buffer** with 29 parts distilled water. Label as **Working Wash Buffer.**

Secondary *Antibody:*

Briefly centrifuge the **100X Secondary Antibody** vial to ensure contents are fully recovered then dilute 1:100 with the **Secondary Antibody Diluent** to create the necessary volume. (e.g. 20 µL stock to 1.98 mL buffer for each 16 wells to be assayed). Label as **Working Secondary Antibody.**

Avidin-HRP Conjugate:

Briefly centrifuge the **100X Avidin-HRP Conjugate** vial to ensure contents are fully recovered then dilute 1:100 with the **Avidin-HRP Diluent** to create the necessary volume. (e.g. 20 µL stock to 1.98 mL diluent for each 16 wells to be assayed). Label as **Working Avidin-HRP.**

TMB Substrate:

TMB Substrate is provided ready for use. To avoid contamination, we recommend transferring the necessary volume to a separate container just prior to use. Do not dump the excess back into original container.

Sample Handling/Preparation:
The primary usefulness of this assay is expected to be in measuring KEAP1 levels in tissue homogenates and cell lysates.

Tissue:
The quantity of tissue required will vary by investigational model system and this information is recommended only as a general guide. Remove excess blood by rinsing tissue in ice-cold PBS (0.02mol/L, pH 7.0-7.2). Homogenize tissue in ice cold PBS using a glass homogenizer. It might also be necessary to sonicate the resulting homogenate to ensure cell membranes are suitably disrupted to allow for detection of KEAP1. Centrifuge the homogenates and harvest the clarified supernatant to be assayed. We have not fully verified the stability of KEAP1 after processing in this manner and recommend testing freshly harvested and processed samples whenever possible. If unable to test immediately, we recommend freezing at -80 °C

Cells
We recommend that cells be disrupted in a similar fashion as tissue, using a glass homogenizer in conjunction with sonication if necessary. Multiple freeze thaw cycles may also be used to disrupt the cells. We do not recommend using commercially available lysis buffers.

Standard Curve Preparation:
Reconstitute the Standard to 10 ng/mL by adding 1mL of **Standard Diluent** into the standard protein vial containing lyophilized mouse KEAP1. Swirl or mix gently, and allow to sit for 5 minutes to ensure complete reconstitution. Use or freeze within 1 hour of reconstitution.

1. Label tubes 1-7 as:
5, 2.5, 1.25, 0.625, 0.312, 0.156 and zero (0) ng/mL.

2. Add 500 µL **Standard Diluent** to each of tubes 1-7.

3. Add 500 µL Reconstituted stock 10 ng/mL Standard to tube 1 and mix well. Tube 1 is now 5 ng/mL.
Note: Unused reconstituted standard can be frozen at -70 °C and thawed one time only without significant loss of immunoreactivity.

4. Make a serial dilution by transferring 500 µL of 5 ng/mL Standard (tube 1) into tube 2 mixing thoroughly then 500 µL of resulting 2.5 ng/mL to tube 3 and so on through to tube 6 to create all Standards down to 0.156 ng/mL.

Assay Protocol:

Standard Procedure for Microplate Assay

1. Bring all reagents to room temperature.

2. Remove appropriate number of wells for assay from foil pouch.

3. Construct an assay template to ensure proper sample addition.

4. Add 100µL of **Sample** or **Standard** to each well to be assayed and 100 µL PBS to Blank wells.

5. Cover with Plate Seal then incubate 2 Hours at 37 °C.

6. Empty contents of wells into sink but do not wash the plate.

7. Add 100µL **Working Secondary Antibody** to each well to be assayed except blank wells.

8. Cover with Plate Seal then incubate for 1 Hour at 37 °C.

9. Empty contents from each well into sink and blot on paper towel to remove as much fluid as possible.

10. Wash plate 3 times as follows:
Add 350µL **Working Wash Buffer** per well
Empty wash solution into sink then blot plate against clean paper towel to remove any remaining wash buffer.

11. Add 100µL of **Working Avidin-HRP** per well.

12. Cover with Plate Seal then incubate at 37°C for 30 minutes.

13. Empty contents of wells into sink and blot on paper towel.

14. Wash the plate 5 times as in Step 10.

15. Add 90µL of **TMB Substrate** per well.

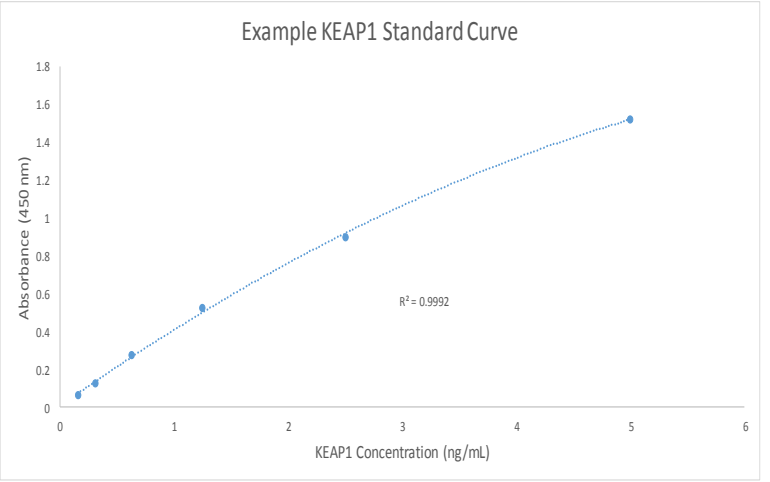
16. Cover with Plate Seal then incubate at 37°C for 15 - 25 minutes. Blue color development should be monitored and the reaction stopped before the highest standard becomes too dark (typically, an Abs 650 of around 1.2 will produce good results in the 450 nm range after stopping).

17. Add 50µL of **Stop Solution** per well.

18. Measure the absorbance at 450nm.

Data Analysis:

Create a standard curve by plotting Absorbance vs. Concentration) for each standard level assayed. If available, set the plate reader to utilize quadratic (2nd order polynomial). An example standard curve is shown below.



Note: Inclusion of the high standard is optional. When samples are found to be in the lower portion of the curve, omission of the high standard can sometimes provide a more accurate curve fit and thus better results.

References:

1. Kubben N, et al. "Repression of the antioxidant NRF2 pathway in premature aging" *Cell* (2016) 165(6):1361-74.

2. Lewis, KN et al. "Regulation of Nrf2 Signaling and longevity in naturally long-lived rodents" *Proc Natl Acad Sci* (2015) Mar 24; 112(12): 3722–3727.

3. Ma Q "Role of Nrf2 in oxidative stress and toxicity" *Pharmacology and Toxicology (2013) Vol. 53: 401-426.*

4. Melba C et al. "The emerging role of the Nrf2–Keap1 signaling pathway in cancer" *Genes & Dev* (2013) 27:2179-2191.

5. Sporn, MB "NRF2 and cancer: the good, the bad and the importance of context" *Nature Reviews Cancer* (2012) 12, 564-571.

6. Taguchi K et al. "Molecular mechanisms of the Keap1–Nrf2 pathway in stress response and cancer evolution" *Genes Cells*, 16 (2011), pp. 123–140.

References (continued):
7. Nguyen,T et al. "The Nrf2-Antioxidant Response Element signaling pathway and its activation by oxidative stress." *J Biol Chem* (2009) 284(20): 13291–13295.

8. Kobayashi A et al. "Oxidative and Electrophilic Stresses Activate Nrf2 Through Inhibition of Ubiquitination Activity of Keap1" *Mol Cell Biol.* 2006 January; 26(1): 221–229.

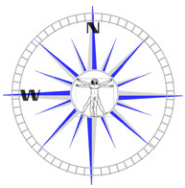
9. Katsuoka F et al. "Genetic Evidence that Small Maf Proteins Are Essential for the Activation of Antioxidant Response Element-Dependent Genes" *Mol Cell Biol.* 2005 September; 25(18): 8044–8051.

10. Kobayashi A et al. "Oxidative Stress Sensor Keap1 Functions as an Adaptor for Cul3-Based E3 Ligase To Regulate Proteasomal Degradation of Nrf2" *Mol Cell Biol.* 2004 August; 24(16): 7130–7139.

Statement of Limited Warranty:
Northwest Life Science Specialties, LLC (NWLSS) makes no guarantee of any kind, expressed or implied, that extends beyond the description of the material in this kit, except that they will meet our specifications at the time of delivery. Customer's remedy and NWLSS' sole liability is limited to, at NWLSS' option, refund of the purchase price, or the replacement of material not meeting our specification. By acceptance of our product, customer assumes all liability and will indemnify and hold NWLSS harmless for the consequence of this product's use or misuse by the customer, its employees, or others. Refund or replacement is conditioned of customer notifying NWLSS within twenty-one (21) days of the receipt of product. Failure to give notice within 21 days shall constitute a waiver by the customer of all claims hereunder with respect to said product.

Notes:

Notes:



Northwest
Life Science Specialties, LLC

5131 NE 94th Avenue, Suite 201
Vancouver, WA 98662
Phone 360-449-3091 or Toll Free: 888-449-3091
Fax 360-449-3092
E-mail: sales@nwlifescience.com