MANUAL

Technology: AlphaLISA®

# AlphaLISA Human KEAP1 Binding Kit

| Part number: | AL3194C | AL3194F |
|---|---|---|
| Assay points: | 500 | 5000 |

Storage: Store kit in the dark at 4 °C. For reconstituted components, aliquot and store at ≤ -20 °C. Avoid freeze-thaw cycles.

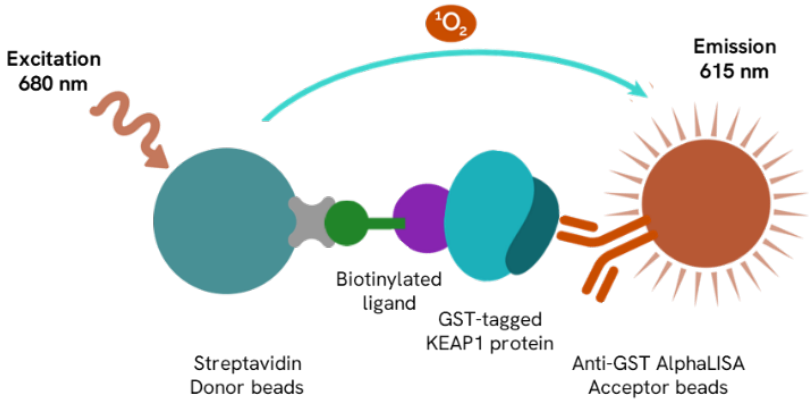
Version: 1 Date: August 2023

## ANALYTE OF INTEREST

The KEAP1-NRF2-ARE pathway plays an important role in response to oxidative stress and maintaining the redox homeostasis.
KEAP1 (Kelch-like ECH-associated protein 1) is an adaptor subunit of Cullin 3-based E3 ubiquitin ligase involved in oxidative stress response. By promoting UPS mediated degradation, KEAP1 controls the expression level of NRF2, a key transcription factor which regulates the expression of antioxidant proteins, therefore ensuring cells protection from oxidative stress. Sustained activation of NRF2 has been associated to cancers resistance, and its inhibition has been shown to dampen drugs resistance. Targeted protein degradation (TPD) uses small molecules to recruit E3 ubiquitin ligases into the proximity of the targeted protein of interest, promoting its ubiquitination-dependent degradation. Exploiting KEAP1 inhibitors for the development of bifunctional Proteolysis-targeting chimeras (PROTACs) is expected to expand the toolbox of E3 ligases to removing undesired proteins (e.g., NRF2) involved in various diseases such as cancers, neurodegenerative diseases, and metabolic disorders.

## DESCRIPTION OF THE ALPHALISA ASSAY

The AlphaLISA detection of KEAP1 binding uses anti-GST AlphaLISA acceptor beads to capture the GST-tagged KEAP1 protein and Streptavidin-coated donor beads to capture the biotinylated ligand. Donor beads and acceptor beads come into proximity through ligand binding to KEAP1 protein. Excitation of the donor beads leads to the release of singlet oxygen that triggers a cascade of energy transfer reactions in the Acceptor beads, resulting in a sharp peak of light emission at 615 nm.




## PRECAUTIONS

- The Alpha Donor beads are light-sensitive. All the other assay reagents can be used under normal light conditions. All Alpha assays using the Donor beads should be performed under subdued laboratory lighting (< 100 lux). Green filters (LEE 090 filters) can be applied to light fixtures.
- Take precautionary measures to avoid contamination of the reagent solutions.
- All blood components and biological materials should be handled as potentially hazardous.

# KIT CONTENT: REAGENTS AND MATERIALS

| Kit components | AL3194C*** | AL3194F*** |
|---|---|---|
| AlphaLISA Anti-GST Acceptor beads stored in PBS, 0.05% Kathon CG/ICP II, pH 7.2 | 40 µL @ 5 mg/mL (1 brown tube, white cap) | 400 µL @ 5 mg/mL (1 brown tube, white cap) |
| Streptavidin (SA)-coated Donor beads stored in 25 mM HEPES, 100 mM NaCl, 0.05% Kathon CG/ICP II, pH 7.4 | 40 µL @ 5 mg/mL (1 brown tube, black cap) | 2 X 1 mL @ 5 mg/mL (x brown tubes, black caps) |
| Lyophilized Biotinylated KEAP1 Ligan* | 13.7 ng (1 tube, clear cap) | 13.7 ng (10 tube, clear cap) |
| Lyophilized KEAP1 protein (GST tagged) | 4.9 µg (1 tube, clear cap) | 4.9 µg (10 tube, clear cap) |
| Lyophilized KEAP1 Standard (Ki696, AL3194S)* | 4.4 µg (1 tube, clear cap) | 4.4 µg (2 tube, clear cap) |
| AlphaLISA Binding Assay Buffer (10X)** | 10 mL, 1 medium bottle | 100 mL, 1 large bottle |

Reconstitute Reconstitute biotinylated KEAP1 ligand, KEAP1 protein and Standard (AL3194S) in 100 µL Milli-Q® grade $H_2O$. IMPORTANT: do not vortex. The reconstituted components should be used within 60 minutes.

Reconstituted biotinylated KEAP1 ligand is stable for 6 weeks if aliquoted and stored at ≤-20°C. Avoid freeze-thaw cycles. Reconstituted KEAP1 protein is stable for 6 weeks if aliquoted and stored at ≤-20°C. Avoid freeze-thaw cycles. Reconstituted KEAP1 Standard is stable for 4 weeks if aliquoted and stored at ≤-20°C. Avoid freeze-thaw cycles.

*One vial contains an amount of standard efficient for performing 10 curves. Additional vials can be ordered separately (cat # AL3194S).

** Extra buffer can be ordered separately (cat # AL018C: 10 mL, cat # AL109F: 100 mL).

*** The number of assay points is based on an assay volume of 20 µL in 384-well assay plates using the kit components at the recommended concentrations.

Sodium azide should not be added to the stock reagents. High concentrations of sodium azide (> 0.001% final in the assay) might decrease the AlphaLISA signal.

## Additional reagents and materials:

The following materials are recommended but not provided in the kit:

| Item | Suggested source |
|---|---|
| Light gray AlphaPlate™-384 | Revvity Inc. |
| TopSeal™-A Plus Adhesive Sealing Film | Revvity Inc. |
| EnVision®-Alpha Reader | Revvity Inc. |

## RECOMMENDATONS

IMPORTANT: PLEASE READ THE RECOMMENDATIONS BELOW BEFORE USE

- The volume indicated on each tube is guaranteed for single pipetting. Multiple pipetting of the reagents may reduce the theoretical amount left in the tube. To minimize loss when pipetting beads, it is preferable not to pre-wet the tip.
- Centrifuge all tubes before use to improve recovery of content (2000*g*, 10-15 sec).
- Re-suspend the Acceptor and Donor beads by vortexing before use.
- Use Milli-Q® grade $H_2O$ to dilute buffers and to reconstitute the lyophilized components. Do not vortex the lyophilized Protein once reconstituted.
- When reagents are added to the microplate, make sure the liquids are at the bottom of the well.
- Small volumes may be prone to evaporation. It is recommended to cover microplates with TopSeal-A Adhesive Sealing Film to reduce evaporation during incubation. Microplates can be read with the TopSeal-A Film in place.
- The AlphaLISA signal is detected with an EnVision Multimode Plate Reader equipped with the Alpha option using the AlphaScreen standard settings (e.g. Total Measurement Time: 550 ms, Laser 680 nm Excitation Time: 180 ms, Mirror: D640as, Emission Filter: M570w, Center Wavelength 570 nm, Bandwidth 100 nm, Transmittance 75%).
- AlphaLISA signal will vary with temperature and incubation time. For consistent results, identical incubation times and temperature should be used for each plate. It is recommended to avoid multiple reads of the same well of the assay plate.

## ASSAY PROCEDURE

- The protocol described below is an *example* for generating 36 assay points in a 20 µL final assay volume per point. If a different number of samples are tested, the volumes of all reagents must be adjusted accordingly. These calculations do not include excess reagent to account for losses during transfer of solutions or dead volumes.
- The dilution protocol is provided for information only. As needed, the number of replicates or the range of concentrations covered can be modified.

The 1-Step protocol is described below. *If different amounts of samples are tested, the volumes of all reagents must be adjusted accordingly.*

1) Preparation of 1X AlphaLISA Binding Assay Buffer:
   Add 1 mL of 10X AlphaLISA Binding Assay buffer and 9 mL Milli-Q® grade $H_2O$.

2) Preparation of AlphaLISA human KEAP1 Binding Kit standard (AL3194S) dilutions:
   a. Reconstitute lyophilized Ki696 Standard (4.4 µg) in 100 µL Milli-Q® grade $H_2O$ to get a 80µM stock solution. The remaining reconstituted standard should be aliquoted immediately and stored at -20 °C (or -80°C) for future assays (see page 2 for more details).

b. Prepare standard dilutions in 1X AlphaLISA Binding Assay Buffer as follows (change tip between each standard dilution).

| Tube | Vol. of Ki696 standard (µL) | Vol. of Buffer (µL)* | [Ki696 Standard] | |
|---|---|---|---|---|
| | | | (µM) 4X | (µM) 1X |
| A | 10 µL of 80µM stock solution | 190 | 4,000 | 1,000 |
| B | 60 µL of tube A | 140 | 1200 | 300 |
| C | 60 µL of tube B | 120 | 400 | 100 |
| D | 60 µL of tube C | 140 | 120 | 30 |
| E | 60 µL of tube D | 120 | 40 | 10 |
| F | 60 µL of tube E | 140 | 12 | 3 |
| G | 60 µL of tube F | 120 | 4 | 1 |
| H | 60 µL of tube G | 140 | 1 | 0.25 |
| I | 60 µL of tube H | 120 | 0.4 | 0.1 |
| J | 60 µL of tube I | 140 | 0.12 | 0.03 |
| K | 60 µL of tube J | 120 | 0.04 | 0.01 |
| L | 0 | 140 | 0 | 0.0 |

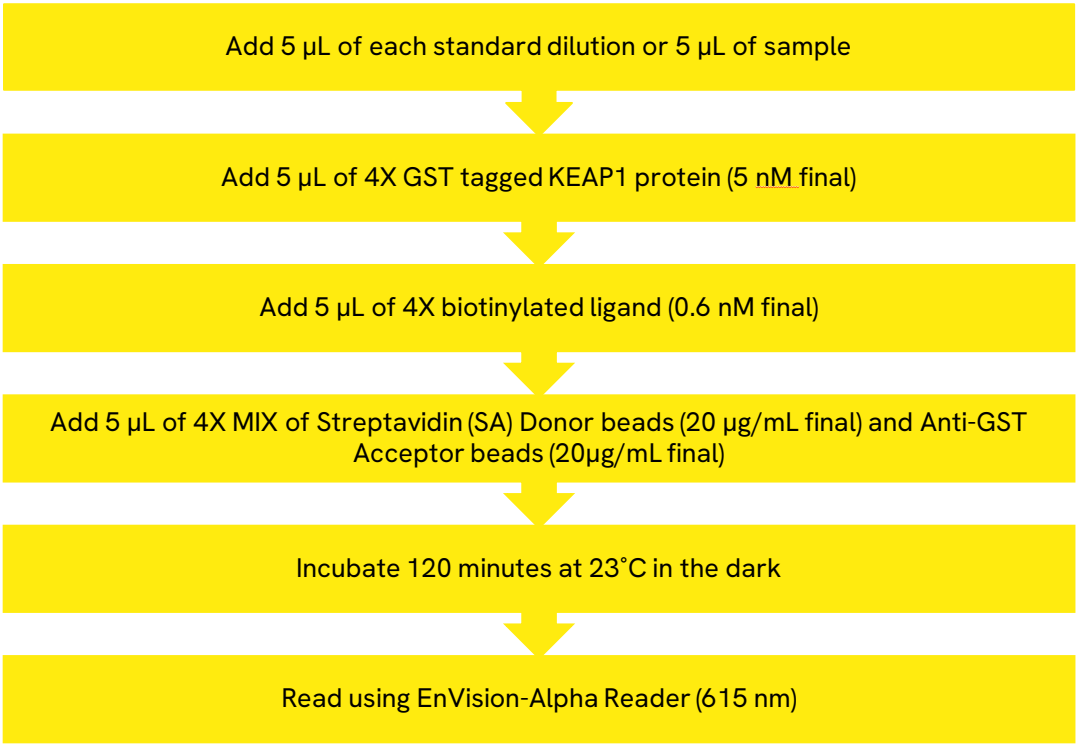
*At low concentrations of analyte, a significant amount of analyte can bind to the vial. Therefore, load the standard dilutions in the assay microplate within 60 minutes of preparation.

3) Preparation of 4X GST tagged KEAP1 protein (20 nM)
   a. Reconstitute lyophilized GST tagged KEAP1 protein in 100 µL Milli-Q® grade $H_2O$ to make a 500nM stock solution. IMPORTANT: do not vortex.
   b. Add 10 µL of the 500 nM KEAP1 stock solution to 240 µL 1X AlphaLISA Binding Assay buffer to obtain a 20nM stock solution of biotinylated ligand.
   c. Prepare just before use.

4) Preparation of 4X KEAP1-biotin ligand (2.4 nM)
   a. Reconstitute lyophilized Biotinylated KEAP1 ligand in 100 µL Milli-Q® grade $H_2O$ to make a 60nM stock solution.
   b. Add 10 µL of 60 nM of Biotin KEAP1 ligand to 240 µL µL 1X AlphaLISA Binding Assay Buffer to obtain 2.4 nM stock solution of biotinylated ligand.
   c. Prepare just before use.

5) Preparation of a 4X MIX of Anti-GST Acceptor beads (80 µg/mL) and 4X Streptavidin (SA) Donor beads (80 µg/mL):
   a. Keep the beads under subdued laboratory lighting.
   b. Add 4 µL of 5 mg/mL Streptavidin (SA) Donor beads and 4 µL of 5 mg/mL Anti-GST Acceptor beads to 242 µL of 1X AlphaLISA Binding Assay Buffer.
   c. Prepare just before use.

5) In a shallow well AlphaPlate (384 wells):

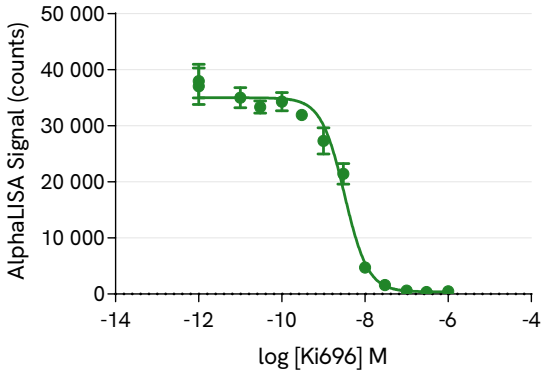



Read settings: AlphaLISA signal is detected using an EnVision Multilabel Reader equipped with the Alpha option using the following settings: Total Measurement Time: 550 ms, Laser: 680 nm, Excitation Time: 180 ms, Mirror: 640as (Barcode# 444), Emission Filter: Wavelength 570nm, bandwidth: 100nm, Transmittance 75%, (Barcode# 244).

## ASSAY PERFORMANCE

### Standard curve:

A typical competition curve of the AlphaLISA Human KEAP1 Binding Kit is shown below, using the Ki696 standard (ref AL3194S) and the 1-step protocol described on pages 4-5. An IC50 of 3.2 nM was calculated by using a nonlinear regression fitting with GraphPad Prism.

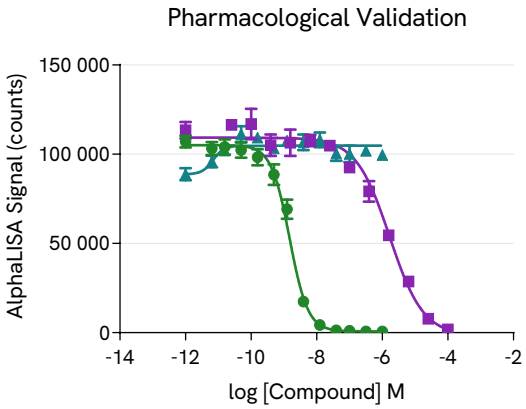



## Pharmacological characterization:

Orthosteric KEAP1 compounds such as Ki696 and ML334 as well as allosteric KEAP1 compounds such as Bardoxolone were characterized according to the 1-step protocol described on pages 4-5.

IC50 indicated in the graph were determined using a nonlinear regression fitting with GraphPad Prism

Potency and pharmacological ranking of Ki696, ML334 are consistent with the literature, whereas the allosteric ligand, Bardoxolone, does not compete with the KEAP1 ligand




| | IC50 |
|---|---|
| KI696 | 1.491e-009 |
| ML334 | 1.676e-006 |
| Bardoxolone | No effect |

# TROUBLESHOOTING

You will find below recommendations for common situations that you might encounter with your AlphaLISA binding assay. If further assistance is needed, do not hesitate to contact our technical support team for assistance or visit us at www.revvity.com.

| Issue | Recommendations and Comments |
|---|---|
| High Background Signal | • Buffer is not freshly made. Make new.<br>• Incubation time is longer than recommended range. |
| Low AlphaLISA Signal | • Optimize EnVision with Plate format |
| High variation between replicates or low Z′ values | • Make sure that reagents are at the bottom of the well by tapping or swirling the plate gently on a smooth surface after each addition |



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