MANUAL

Technology: AlphaLISA®

AlphaLISA RIG-I Binding Kit

Part number:	AL3192C	AL3192F
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.

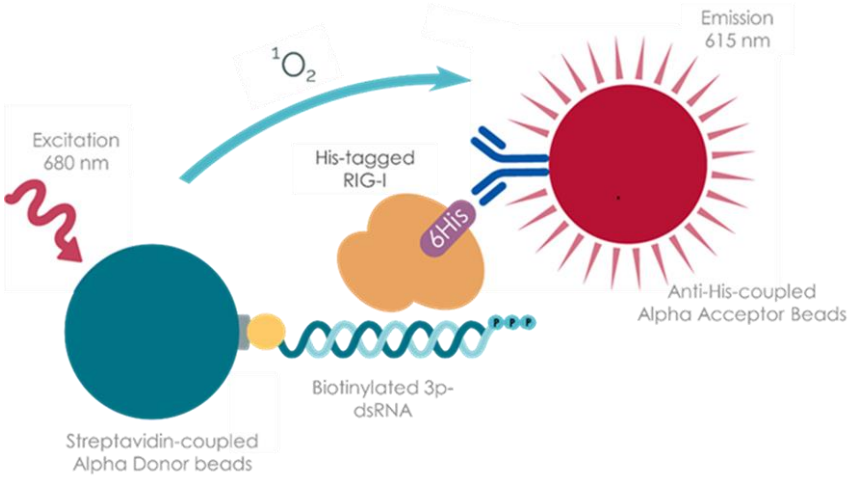
Version: 1 Date: August 2023

ANALYTE OF INTEREST

A fast and easy way to identify new binders to human RIG-I. The Retinoic acid-Inducible Gene I (RIG-I) is a cytosolic pattern recognition receptor which is known to recognize RNA pathogens in cells. This protein is involved in the innate immune system and induces a type 1 interferon response. The RIG-I protein recognizes infected cells by detecting viral dsRNA. Once activated by a dsRNA, the N-terminus domain of RIG-I binds mitochondrial antiviral signaling proteins, thereby activating the IFN-1 signaling pathway.

DESCRIPTION OF THE ALPHALISA ASSAY

The AlphaLISA RIG-I Binding Kit uses Anti-6His AlphaLISA Acceptor beads to capture the RIG-tagged protein and Streptavidin-coated Donor beads to capture the biotinylated-3P dsRNA. Donor beads and Acceptor beads come into proximity through RIG-I binding to 3P-dsRNA. Excitation of the Donor beads provokes the release of singlet oxygen that triggers a cascade of energy transfer reactions within the Acceptor beads, resulting in emission with λ_{max} 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	AL3192C***	AL3192F***
AlphaLISA Anti-6His 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)	400 µL @ 5 mg/mL (1 brown tubes, black caps)
Lyophilized RIG-I (25X)	0.1 µM (1 tube, clear cap)	0.1 µM (10 tubes, clear cap)
Lyophilized Biotinylated 3P-dsRNA (100X)	0.4 µM (1 tube, clear cap)	0.4 µM (3 tubes, clear cap)
Lyophilized RIG-I Binding kit Standard*	1 µM (1 tube, clear cap)	1 µM (1 tube, clear cap)
AlphaLISA PPI Buffer (5X)**	10 mL, 1 medium bottle	100 mL, 1 large bottle

* Reconstitute RIG-I protein, biotinylated 3S-dsRNA, and standard in 100 µL Milli-Q® grade H_2O. The reconstituted components should be used within 60 minutes.

Reconstituted RIG-I protein is stable for 2 weeks if aliquoted and stored at ≤-20°C . Avoid freeze-thaw cycles. Stability increases to 4 weeks with a storage at ≤-60°. Reconstituted biotinylated 3P-dsRNA is stable for 2 weeks if aliquoted and stored at ≤-20°C . Avoid freeze-thaw cycles. Stability increases to 4 weeks with a storage at ≤-60°. Reconstituted Standard is stable for 2 weeks if aliquoted and stored at ≤-60°C . Avoid freeze-thaw cycles. S/B may highly decrease due to a storage at -20°C.

*One vial contains an amount of standard efficient for performing 10 curves. Additional vials can be ordered separately (cat # AL3192S).

** Extra buffer can be ordered separately (cat # AL015C: 10 mL, cat # AL015F: 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.
- 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 288 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 PPI Buffer:
   Add 2 mL of 5X AlphaLISA PPI Buffer to 8 mL Milli-Q® grade H_2O.

2) Preparation of AlphaLISA human RIG-I Binding Kit standard dilutions (3P-dsRNA):
   a. Reconstitute lyophilized 3P-dsRNA in 100 µL Milli-Q® grade H_2O. The remaining reconstituted standard should be aliquoted immediately and stored at -20 °C for future assays (see page 2 for more details).

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

Tube	Vol. of 3P-dsRNA (µL)	Vol. of diluent (µL)*	[3P-dsRNA]	
			(M)	(pM in 5µL)
A	10 µL of reconstituted 3P-dsRNA)	90	1.00E-07	100 000
B	60 µL of tube A	140	3.00E-08	30 000
C	60 µL of tube B	120	1.00E-08	10 000
D	60 µL of tube C	140	3.00E-09	3 000
E	60 µL of tube D	120	1.00E-09	1 000
F	60 µL of tube E	140	3.00E-10	300
G	60 µL of tube F	120	1.00E-10	100
H	60 µL of tube G	140	3.00E-11	30
I	60 µL of tube H	120	1.00E-11	10
J	60 µL of tube I	140	3.00E-12	3
K	60 µL of tube J	120	1.00E-12	1
L	0	140	1.00E-13	0

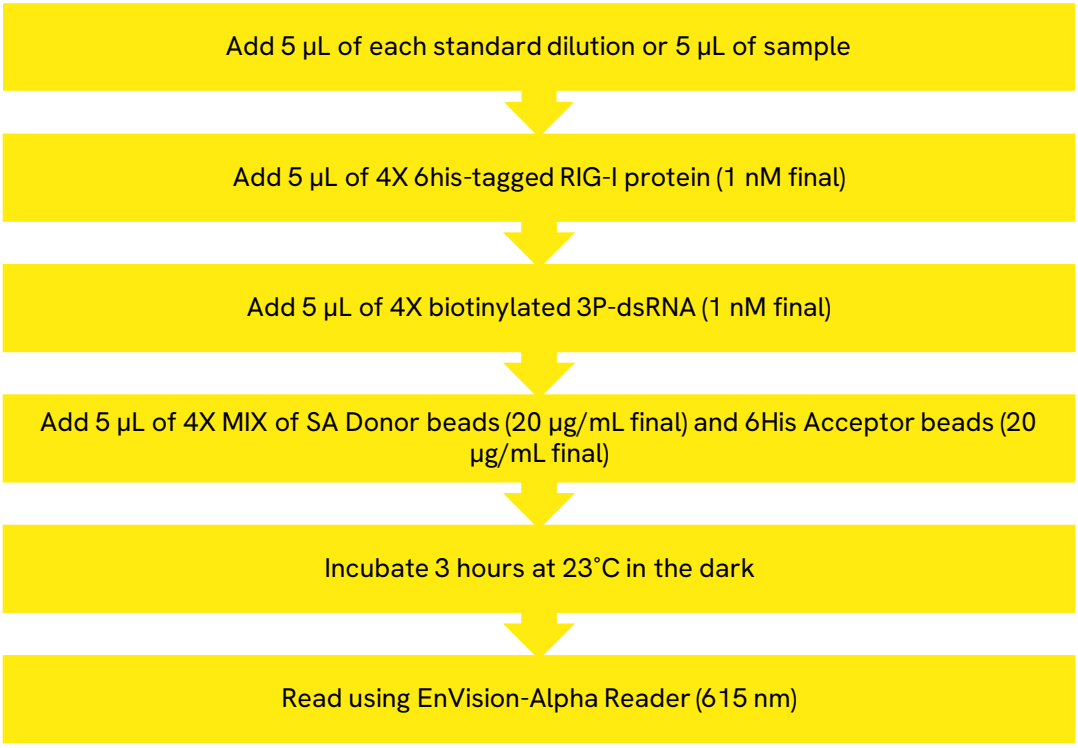
*At low concentrations of analyte, a significant amount of analyte can bind to the vial. Therefore, load the standard dilutions in the assay microplate within 60 minutes of preparation.

3) Preparation of 4X 6His-tagged RIG-I protein (4nM)
   a. Reconstitute lyophilized RIG-I protein in 100 µL Milli-Q® grade H_2O to make a 0.1 µM stock solution.
   b. Add 57.6 µL of 0.1 µM RIG-I protein to 1382.4 µL 1X AlphaLISA PPI buffer to obtain a 4nM stock solution of 6his-tagged RIG-I protein.
   c. Prepare just before use.

4) Preparation of 4X biotinylated 3P-dsRNA (4nM)
   a. Reconstitute lyophilized biotinylated 3P-dsRNA in 100 µL Milli-Q® grade H_2O to make a 0.4 µM stock solution.
   b. Add 14.4 µL of 0.4 µM biotinylated 3P-dsRNA to 1425.6 µL 1X AlphaLISA PPI buffer to obtain a 4nM stock solution of biotinylated ligand.
   c. Prepare just before use.

5) Preparation of a 4X MIX of Streptavidin Donor beads (100µg/mL) and 6His Acceptor beads (100 µg/mL):
   a. Keep the beads under subdued laboratory lighting.
   b. Add 23 µL of 5 mg/mL Streptavidin Donor beads and 23 µL of 5 mg/mL 6His Acceptor beads to 1394 µL of 1X AlphaLISA PPI Buffer.
   c. Prepare just before use.

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

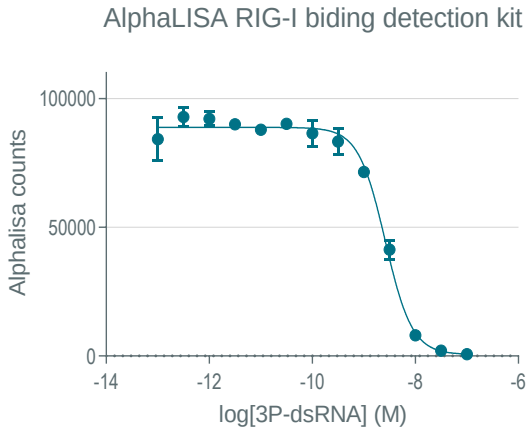


Read settings: AlphaLISA signal is detected using an EnVision Multilabel Reader equipped with the Alpha option using the following settings: Total Measurement Time: 550 ms, Laser: 680 nm, Excitation Time: 180 ms, Mirror: 640as (Barcode# 444), Emission Filter: Wavelength 570nm, bandwidth: 100nm, Transmittance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

ASSAY PERFORMANCE

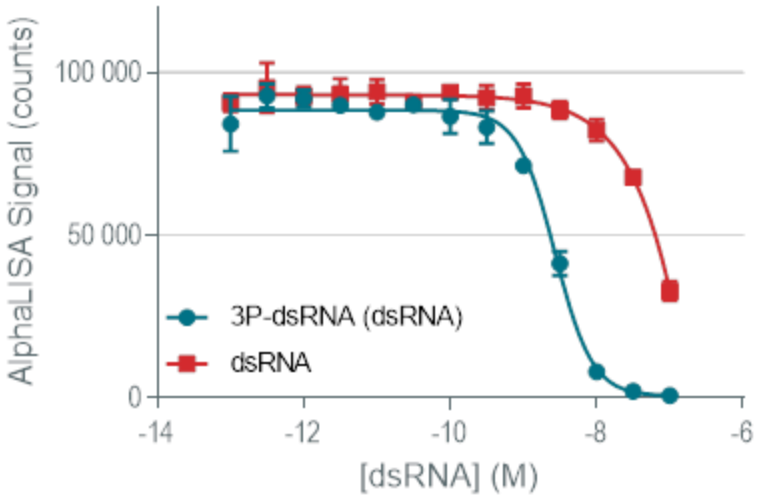
Standard curve:

A typical competition curve of the AlphaLISA RIG-I Binding Kit is shown below, using the 3P-dsRNA standard and the 1-step protocol described on pages 4-5. An IC50 of 2.6 nM was calculated by using a nonlinear regression fitting with GraphPad Prism.



Pharmacological characterization:

3P-dsRNA and ds-RNA were tested in the AlphaLISA RIG-I Binding Kit the 1-step protocol described on pages 4-5. The IC50 for 3P-dsRNA was calculated at 2.7 nM by using a nonlinear regression fitting with GraphPad Prism and is listed to the right of the graph below. As expected, the RIG-I protein have an higher affinity to 3P-dsRNA compared to ds-RNA.



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. • 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

www.revvity.com

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Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA

(800) 762-4000
www.revvity.com

For a complete listing of our global offices, visit www.revvity.com