TECHNICAL DATA SHEET

AlphaLISA® Research Reagents

Research Use Only. Not for use in diagnostic procedures.

p62 (Human) AlphaLISA Detection Kit

Product number: AL3097

Contents

Product Information

Application: This kit is designed for the quantitative determination of human p62 in human cell lysate using a homogeneous AlphaLISA assay (no wash steps).

Sensitivity: Lower Detection Limit (LDL): 20.5 pg/mL

Lower Limit of Quantification (LLOQ): 66 pg/mL

EC_{50}: 140 ng/mL

Dynamic range: Dynamic Range: 20 – 1 000 000 pg/mL

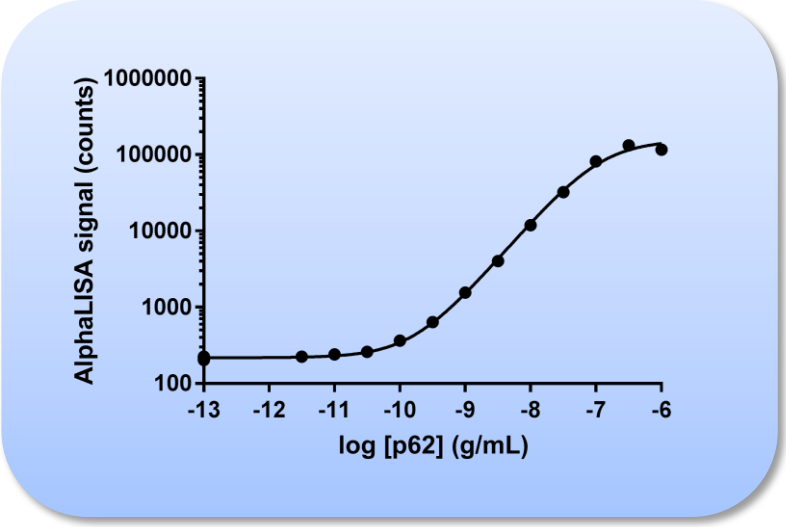


Figure 1. Typical sensitivity curve in AlphaLISA Immunoassay Buffer.

Storage: Store kit in the dark at +4˚C. Store reconstituted analyte at -20 °C. Limit the number of freeze-thaw cycles.

Stability: This kit is stable for at least 6 months from the manufacturing date when stored in its original packaging and the recommended storage conditions.

Quality Control

Lot to lot consistency is confirmed in an AlphaLISA assay. Maximum and minimum signals, EC_{50} and LDL were measured on the EnVision Multilabel Plate Reader with Alpha option using the protocol described in this technical data sheet. We certify that these results meet our quality release criteria. Maximum counts may vary between bead lots and the instrument used, with no impact on LDL measurement.

Analyte of Interest

The ubiquitin binding protein p62 or Sequestosome 1 (SQSTM1) is expressed in most cell types. Synonyms are p62 lck ligand, zeta interacting protein (ZIP, rat), A170 (mouse), OSIL or PDB3. The human protein is 440 amino acids long. It contains an N-terminal protein binding domain (PB1) that interacts with different protein kinases and allows homopolymerization of p62. The domain is followed by several other protein-protein interaction domains and nuclear localization and export signals. Notably, the C-terminal ubiquitin association domain recognizes both mono and polyubiquitin and the LIR motif allows direct binding to the autophagosome-associated protein LC3. p62 plays an important role in the formation of intracellular protein aggregates, autophagy, and NF-κB signaling. Mutation of p62 is associated with Paget's disease (abnormal bone turnover).

Description of the AlphaLISA Assay

This AlphaLISA kit allows the detection of p62 in buffer and cell lysates in a highly sensitive, quantitative, reproducible and user-friendly mode. In the AlphaLISA assay, a Biotinylated Anti-p62 antibody binds to the Streptavidin-coated Alpha Donor beads, while another Anti-p62 antibody is conjugated to AlphaLISA Acceptor beads. In the presence of the analyte, the beads come into close proximity. The excitation of the Donor beads provokes the release of singlet oxygen molecules that triggers a cascade of energy transfer in the Acceptor beads, resulting in a sharp peak of light emission at 615 nm (Figure 2).

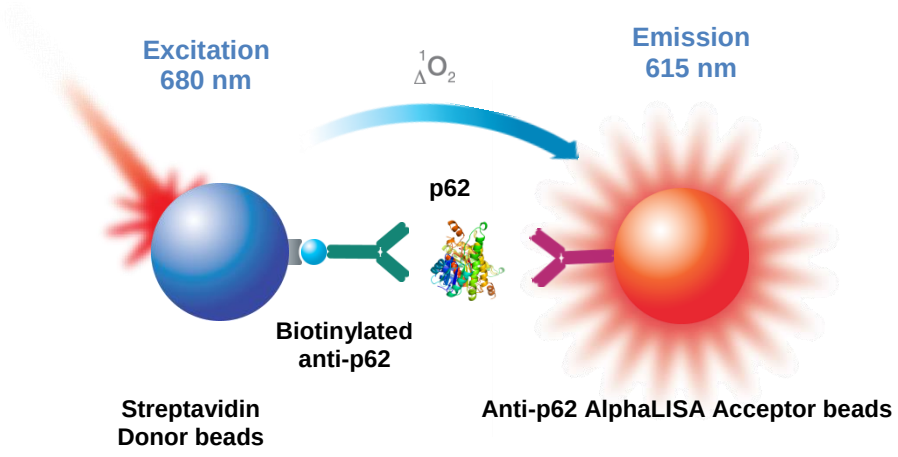


Figure 2. AlphaLISA Assay Principle.

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 (preferred) or Roscolux filters #389 from Rosco) can be applied to light fixtures.
- Take precautionary measures to avoid contamination of the reagent solutions.
- The Biotinylated Anti-Analyte Antibody contains sodium azide. Contact with skin or inhalation should be avoided.

Kit Content: Reagents and Materials

Kit components	AL3097HV (100 assay points***)	AL3097C (500 assay points***)	AL3097F (5000 assay points***)
AlphaLISA Anti-p62 Acceptor beads stored in PBS, 0.05% Proclin-300, pH 7.2	20 µL @ 5 mg/mL (1 brown tube, white cap)	50 µL @ 5 mg/mL (1 brown tube, white cap)	500 µL @ 5 mg/mL (1 brown tube, white cap)
Streptavidin (SA)-coated Donor beads stored in 25 mM HEPES, 100 mM NaCl, 0.05% Proclin-300, pH 7.4	80 µL @ 5 mg/mL (1 brown tube, black cap)	200 µL @ 5 mg/mL (1 brown tube, black cap)	2 X 1 mL @ 5 mg/mL (2 brown tubes, black caps)
Biotinylated Anti-p62 stored in PBS, 0.1% Tween-20, 0.05% NaN_3, pH 7.4	20 µL @ 500 nM (1 tube, black cap)	50 µL @ 500 nM (1 tube, black cap)	500 µL @ 500 nM (1 tube, black cap)
Human p62 Lyophilized Analyte*	1 µg (1 tube, clear cap)	1 µg (1 tube, clear cap)	1 µg (1 tube, clear cap)
AlphaLISA Immunoassay Buffer (10X) **	2 mL, 2 small bottles	10 mL, 2 small bottles	100 mL, 2 large bottles
AlphaLISA Lysis buffer (5X)	2 mL 1 small bottle	10 mL 1 small bottle	100 mL 1 small bottle

* Reconstitute p62 in 100 µL Milli-Q® grade H_2O. Reconstituted analyte may be stored at 4°C for up to 30 days. One vial contains an amount of analyte sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AL3097S).

** Extra buffer can be ordered separately (Immunoassay Buffer: cat # AL000C: 10 mL, cat # AL000F: 100 mL). (Lysis Buffer: cat # AL003C: 10 mL, cat # AL003F: 100 mL).

*** The number of assay points is based on an assay volume of 100 µL in HV size kits or 50 µL in C/F size kits 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. Note that sodium azide from the Biotinylated Antibody stock solution will not interfere with the AlphaLISA signal (0.0001% final in the assay).

Specific additional required reagents and materials:

The following materials are recommended:

Item	Suggested source	Catalog #
TopSeal™-A Plus Adhesive Sealing Film	PerkinElmer Inc.	6050185
EnVision®-Alpha Reader	PerkinElmer Inc.	-

Recommendations

- 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 (including lyophilized analyte) before use to improve recovery of content (2000g, 10-15 sec). Re-suspend all reagents by vortexing before use.
- Use Milli-Q® grade H_2O to dilute 10X AlphaLISA Immunoassay Buffer and to reconstitute the lyophilized analyte.
- When diluting the standard or samples, change tips between each standard or sample dilution. When loading reagents in the assay microplate, change tips between each standard or sample addition and after each set of reagents.
- 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 Plus Adhesive Sealing Films to reduce evaporation during incubation. Microplates can be read with the TopSeal-A Plus Film.
- The AlphaLISA signal is detected with an EnVision Multilabel 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.

- The standard curves shown in this technical data sheet are provided for information only. A standard curve must be generated for each experiment.

Assay Procedure

IMPORTANT: PLEASE READ THE RECOMMENDATIONS BELOW BEFORE USE

- The protocol described below is an example for generating one standard curve in a 50 µL final assay volume (48 wells, triplicate determinations). The protocols also include testing samples in 452 wells. If a different amount of samples are tested, the volumes of all reagents have to be adjusted accordingly, as shown in the table below. These calculations do not include excess reagent to account for losses during transfer of solutions or dead volumes.
- The standard dilution protocol is provided for information only. As needed, the number of replicates or the range of concentrations covered can be modified.
- Use of four background points in triplicate (12 wells) is recommended when LDL/LLOQ is calculated. One background point in triplicate (3 wells) can be used when LDL/LLOQ is not calculated.

			Volume			
Format	**# of data points**	**Final**	**Sample**	**AlphaLISA MIX Acceptor beads + Biotinylated Antibody**	**SA-Donor beads**	**Plate recommendation**
AL3097HV	100	100 µL	10 µL	40 µL	50 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
AL3097C	250	100 µL	10 µL	40 µL	50 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
	500	50 µL	5 µL	20 µL	25 µL	White ½ AreaPlate-96 (cat # 6005560) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate™-384 (cat # 6005350)
	1 250	20 µL	2 µL	8 µL	10 µL	Light gray AlphaPlate-384 (cat # 6005350) ProxiPlate™-384 Plus (cat # 6008280) White OptiPlate-384 (cat # 6007290)
	2 500	10 µL	1 µL	4 µL	5 µL	Light gray AlphaPlate-1536 (cat # 6004350)
AL3097F	5 000	50 µL	5 µL	20 µL	25 µL	White ½ AreaPlate-96 (cat # 6005560) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate-384 (cat # 6005350)
	12 500	20 µL	2 µL	8 µL	10 µL	Light gray AlphaPlate-384 (cat # 6005350) ProxiPlate-384 Plus (cat # 6008280) White OptiPlate-384 (cat # 6007290)
	25 000	10 µL	1 µL	4 µL	5 µL	Light gray AlphaPlate-1536 (cat # 6004350)

2 Step Protocol described below is for 500 assay points including one standard curve (48 wells) and samples (452 wells). *If a different amount of samples are tested, the volumes of all reagents have to be adjusted accordingly.*

1) Preparation of 1X AlphaLISA Immunoassay Buffer:
   a. Add 5 mL of 10X AlphaLISA Immunoassay Buffer to 45 mL H_2O.

2) Preparation of p62 analyte standard dilutions:
   a. Reconstitute lyophilized p62 (1 µg) in 100 µL MilliQ H_2O. The remaining reconstituted analyte should be stored at 4ºC for future assays (see page 4 for more details).
   b. Prepare standard dilutions as follows in 1X AlphaLISA Immunoassay Buffer (change tip between each standard dilution):

Tube	Vol. of p62 (µL)	Vol. of diluent (µL) *	[p62] in standard curve	
			(g/mL in 5 µL)	(pg/mL in 5 µL)
A	10 µL of reconstituted p62	90	1.00E-06	1 000 000
B	60 µL of tube A	140	3.00E-07	300 000
C	60 µL of tube B	120	1.00E-07	100 000
D	60 µL of tube C	140	3.00E-08	30 000
E	60 µL of tube D	120	1.00E-08	10 000
F	60 µL of tube E	140	3.00E-09	3 000
G	60 µL of tube F	120	1.00E-09	1 000
H	60 µL of tube G	140	3.00E-10	300
I	60 µL of tube H	120	1.00E-10	100
J	60 µL of tube I	140	3.00E-11	30
K	60 µL of tube J	120	1.00E-11	10
L	60 µL of tube K	140	3.00E-12	3
M ** (background)	0	100	0	0
N ** (background)	0	100	0	0
O ** (background)	0	100	0	0
P ** (background)	0	100	0	0

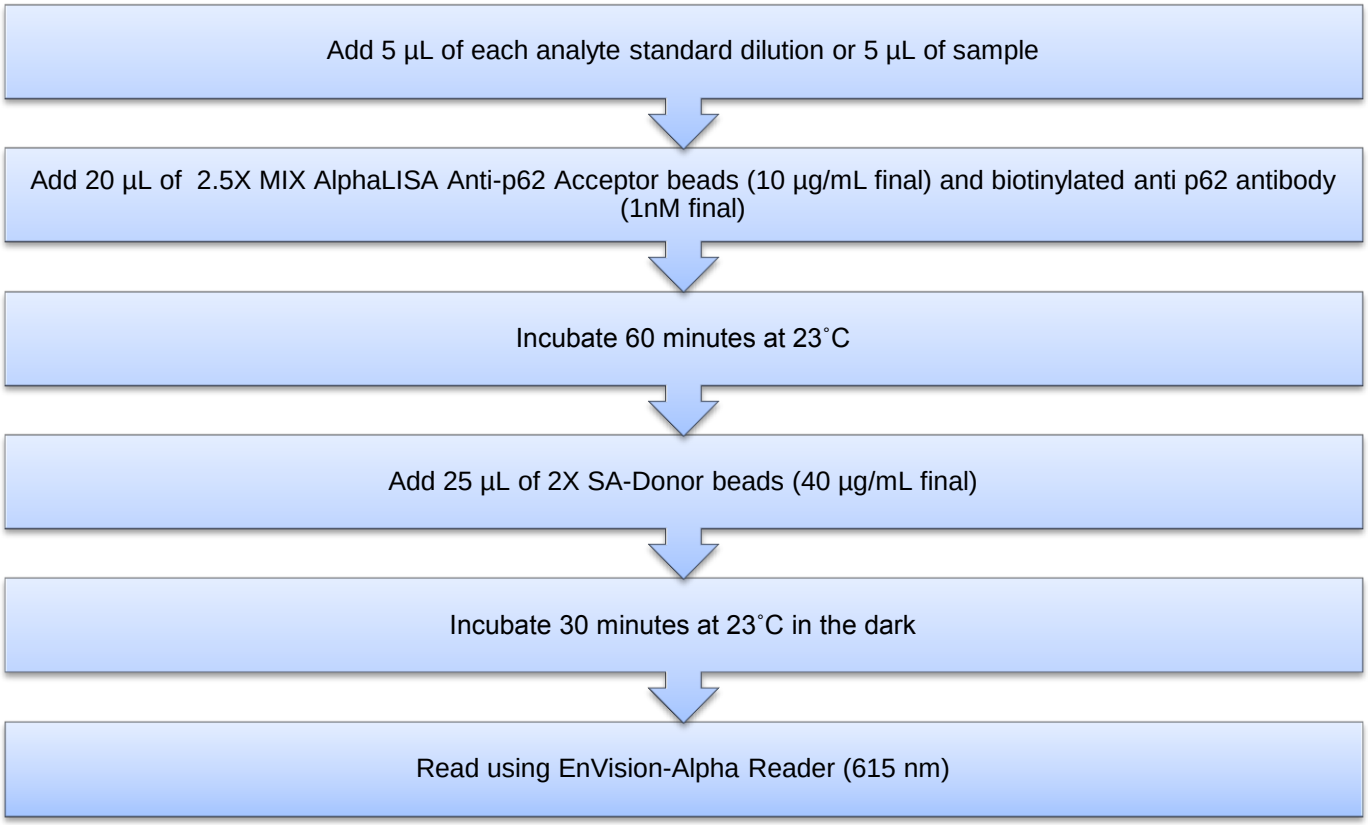
* Dilute standards in diluent (e.g. 1X AlphaLISA Immunoassay Buffer or Lysis buffer).
At low concentrations of analyte, a significant amount of analyte can bind to the vial. Therefore, load the analyte standard dilutions in the assay microplate within 60 minutes of preparation.

** Four background points in triplicate (12 wells) are used when LDL is calculated. If LDL does not need to be calculated, one background point in triplicate can be used (3 wells).

3) Preparation of 2.5X MIX AlphaLISA Anti-p62 Acceptor beads (25 µg/mL) and biotinylated anti-p62 antibody (2.5 nM)
   a. Prepare just before use.
   b. Add 50 µL of 5 mg/mL AlphaLISA Anti-p62 Antibody Acceptor and 50 µL of 500 nM biotinylated Anti-p62 Antibody to 9900 µL of 1X AlphaLISA Immunoassay Buffer.

4) Preparation of 2X Streptavidin (SA) Donor beads (80 µg/mL):
   a. Prepare just before use.
   b. Keep the beads under subdued laboratory lighting.
   c. Add 200 µL of 5 mg/mL SA-Donor beads to 12300 µL of 1X AlphaLISA Immunoassay Buffer.

5) In a white Optiplate (384 wells):



Data Analysis

- Calculate the average count value for the background wells.
- Generate a standard curve by plotting the AlphaLISA counts versus the concentration of analyte. A log scale can be used for either or both axes. No additional data transformation is required.
- Analyze data according to a nonlinear regression using the 4-parameter logistic equation (sigmoidal dose-response curve with variable slope) and a $1/Y^2$ data weighting (the values at maximal concentrations of analyte after the hook point should be removed for correct analysis).
- The LDL is calculated by interpolating the average background counts (12 wells without analyte) + 3 x standard deviation value (average background counts + (3xSD)) on the standard curve.
- The LLOQ as measured here is calculated by interpolating the average background counts (12 wells without analyte) + 10 x standard deviation value (average background counts + (10xSD)) on the standard curve. Alternatively, the true LLOQ can be determined by spiking known concentrations of analyte in the matrix and measuring the percent recovery, and then determining the minimal amount of spiked analyte that can be quantified within a given limit (usually +/- 20% or 30% of the real concentration).
- Read from the standard curve the concentration of analyte contained in the samples.
- If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Assay Performance Characteristics

AlphaLISA assay performance described below was determined using the 2 step protocol in AlphaLISA Immunoassay Buffer.

- Assay Sensitivity:

The LDL was calculated as described above. The values correspond to the lowest concentration of analyte that can be detected in a volume of 5 µL using the recommended assay conditions.

LDL (pg/mL)	Buffer/Medium*	# of experiments
20.5	Immunoassay Buffer	9
129	Lysis buffer	6

- Assay Precision:

The following assay precision data were calculated from the three independent assays using two different kit lots. In each lot, the analytes were prepared in Immunoassay buffer or Lysis buffer. Each assay consisted of one standard curve comprising 12 data points (each in triplicate) and 12 background wells (no analytes). The assays were performed in 384-well format using Immunoassay buffer.

- Intra-assay precision:

The intra-assay precision was determined using 3 independent experiments for a total of 16 independent determinations in triplicate. CV% were calculated for each individual experiment then averaged. Shown is the average intra-experimental CV%.

p62	IAB	Lysis
CV (%)	5	3

- Inter-assay precision:

The inter-assay precision was determined using the data across 3 independent experiments with 16 measurements in triplicate. CV% was calculated by comparing the same measurement in each experiment. The CV% for all 16 measurements was then averaged. Shown is the inter-experimental CV%.

p62	IAB	Lysis
CV (%)	5	6

- Spike Recovery:

Known concentrations of analyte were spiked into IAB or Lysis Buffer. All samples, including non-spiked buffer or media were measured in the assay. Note that the standard curves were prepared in Immunoassay or Lysis Buffer.

Spiked p62 (µg/mL)	Immunoassay	Lysis buffer
1	88	94
0.1	106	110
0.01	113	118

- Specificity:

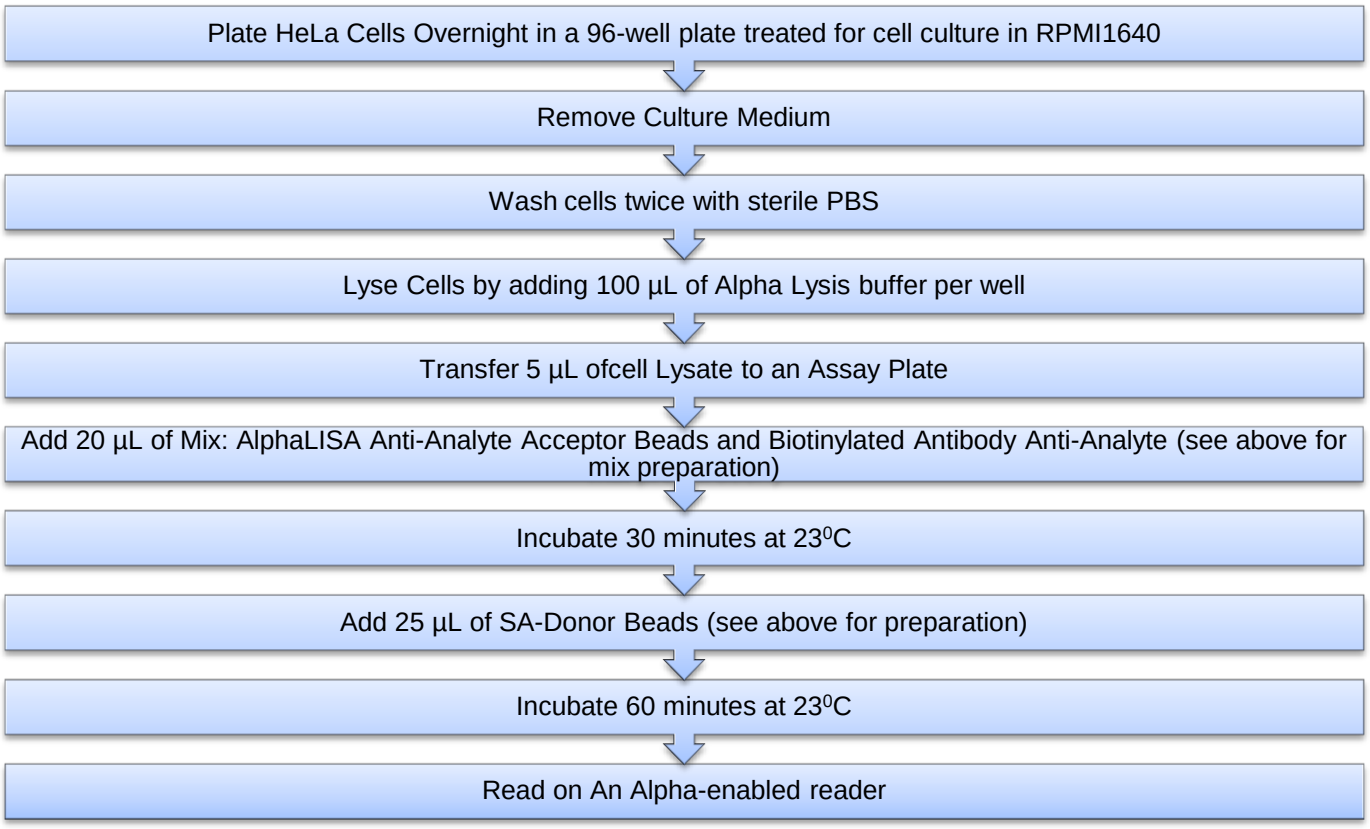
Cross-reactivity of the AlphaLISA p62 Kit was tested using the following protein at 3 µg/mL in AlphaLISA Immunoassay Buffer.

Protein	% Cross-reactivity
Next to BRCA1 gene 1 protein (NBR1)	106

The possible interference from LC3B, containing a His-tag at the N-terminus, was investigated. The p62 was kept at a constant concentration (EC50 value of the standard curve). The binding protein was titrated into the assay. No interference was observed up to 10 µg/mL, which is the maximum concentration tested.

Cell Lysates Assay

Cell lysate assays were performed with non-stimulated HeLa cells grown in RPMI1640. The following protocol was used:

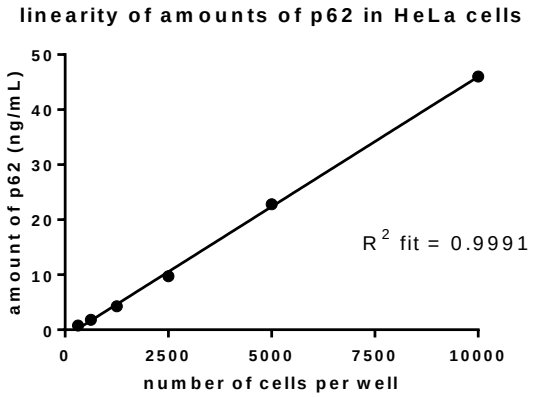


Typical results

• Dilutional linearity:

The dilutional linearity was determined by measuring the amount of p62 in wells bearing various amounts of HeLa cells starting at 10 000 cells per well and diluting by factors of 2. The results were plotted on a standard curve performed in AlphaLISA Lysis buffer.

Cells per well	p62 (ng/mL)



10 000	46.0
5 000	22.8
2 500	9.72
1 250	4.38
625	1.83
313	0.79

Troubleshooting Guide

You will find detailed recommendations for common situations you might encounter with your AlphaLISA Assay kit at:

http://www.perkinelmer.com/in/resources/technicalresources/applicationsupportknowledgebase/alphalisa-alphascreen-no-washassays/alpha_troubleshoot.xhtml

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