TECHNICAL DATA SHEET

AlphaLISA® Research Reagents

Research Use Only. Not for use in diagnostic procedures

Bruton Tyrosine Kinase (BTK) (Human) AlphaLISA Detection Kit

Product number: AL3110

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Product Information

Application: This kit is designed for the quantitative determination of Human BTK in buffer and cell lysates, using a homogeneous AlphaLISA assay (no wash steps).

Sensitivity: Lower Detection Limit (LDL): 24 pg/mL

Lower Limit of Quantification (LLOQ): 143 pg/mL

EC_{50}: 195 ng/mL

Dynamic range: 24 – 300 000 pg/mL (Figure 1).

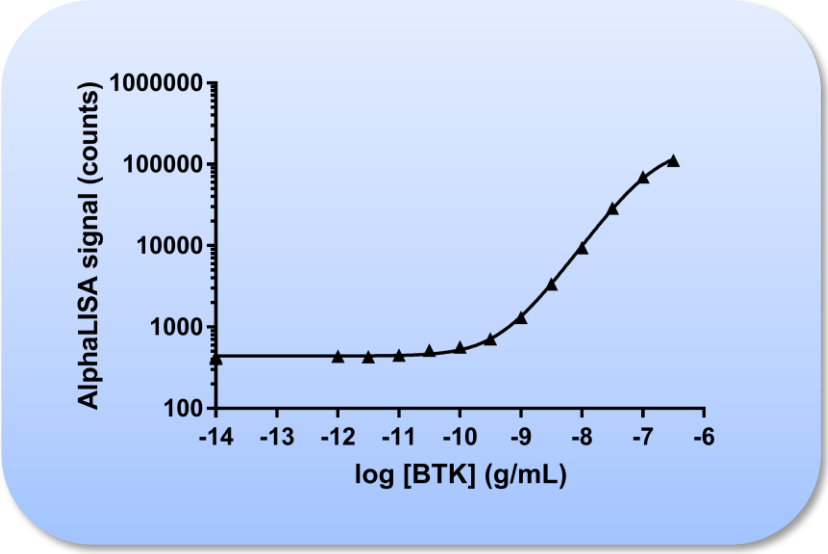


Figure 1. Typical sensitivity curve with standard in AlphaLISA Lysis Buffer and other reagents prepared in AlphaLISA Immunoassay Buffer. The data was generated using a white Optiplate™-384 microplate and the EnVision® Multilabel Plate Reader 2102 with Alpha option.

Storage: Store kit in the dark at +4˚C.

Stability: The analyte is shipped on dry ice and is stable for at least 3 months at -80 ^{0}C. The rest of the kit is stable for at least 12 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

BTK is a cytoplasmic tyrosine kinase present in B-cells. It is associated with the B-cell receptor and receptors of the toll family. Upon activation, BTK will activate the Nf-kB pathway. This will induce maturation of B-cells into antibody producing cells and also increase the secretion of activating cytokines (such as IL-2). The protein is absent or non-functional in diseases such as aglobularia, but is also involved by hyperactivation in inflammation and auto-immune diseases. Also, several leukemia-like cancers have dysregulation of BTK.

Description of the AlphaLISA Assay

AlphaLISA technology allows the detection of BTK in buffer and cell lysate in a highly sensitive, quantitative, reproducible and user-friendly mode. In an AlphaLISA assay, a Biotinylated Anti-BTK Antibody binds to the Streptavidin-coated Alpha Donor beads, while another Anti-BTK 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).

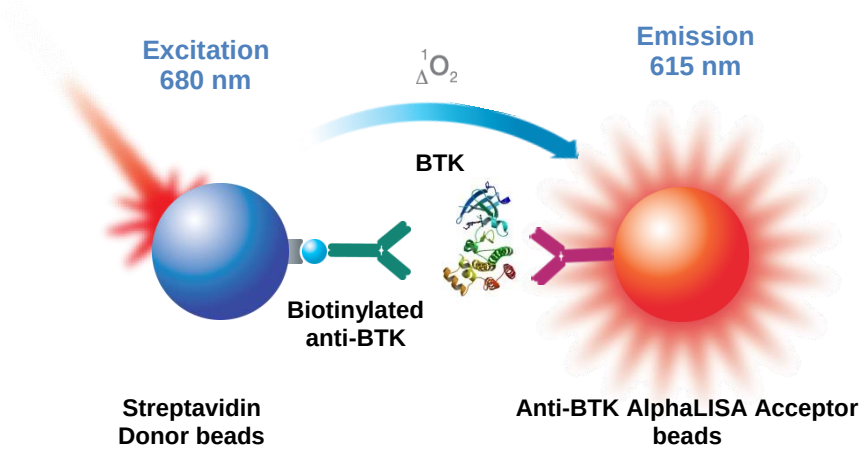


Figure 2. AlphaLISA Assay Principle.

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.
- All blood components and biological materials should be handled as potentially hazardous. The analyte included in this kit is from a human source.
- Some analytes are present in saliva. 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	AL3110HV (100 assay points)	AL3110C (500 assay points***)	AL3110F (5000 assay points***)
AlphaLISA Anti-hBTK 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)	2x 1mL @ 5 mg/mL (1 brown tube, black cap)
Biotinylated Anti-hBTK 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)
BTK Analyte* (store as -80^{0}C)	100 µL @ 3 µg/mL (1 tube, clear cap)	100 µL @ 3 µg/mL (1 tube, clear cap)	100 µL @ 3 µg/mL (1 tube, clear cap)
AlphaLISA Immunoassay Buffer (10X) **	2 mL, 1 small bottle	10 mL, 1 small bottle	100 mL, 1 large bottle
AlphaLISA Lysis Buffer (5X) **	2 mL, 1 small bottle	10 mL, 1 small bottle	100 mL, 1 large bottle

* Remaining analyte should be aliquoted and stored at -80^{0}C for further experiments. Avoid multiple freeze-thaws. Analyte aliquoted is stable for at least 3 months at -80^{0}C. One vial contains an amount of hBTK sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AL3110S).

** 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 50 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. 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	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 (18 MΩ•cm) to dilute 10X AlphaLISA Immunoassay Buffer.
- 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 Adhesive Sealing Films to reduce evaporation during incubation. Microplates can be read with the TopSeal-A 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. The standard curve should be performed in 10% FBS in AlphaLISA Immunoassay Buffer for plasma samples and in Alpha lysis buffer for cell lysates.

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 Acceptor beads and Biotinylated Antibody**	**SA-Donor beads**	**Plate recommendation**
AL3110HV	100	100 µL	10 µL	10 µL	80 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
AL3110C	250	100 µL	10 µL	10 µL	80 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
	500	50 µL	5 µL	5 µL	40 µL	White ½ AreaPlate-96 (cat # 6005560) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate™-384 (cat # 6005350)
	1 250	20 µL	2 µL	2 µL	16 µL	Light gray AlphaPlate-384 (cat # 6005350) ProxiPlate™-384 Plus (cat # 6008280) White OptiPlate-384 (cat # 6007290)
	2 500	10 µL	1 µL	1 µL	8 µL	Light gray AlphaPlate-1536 (cat # 6004350)
AL3110F	5 000	50 µL	5 µL	5 µL	40 µL	White ½ AreaPlate-96 (cat # 6005560) White OptiPlate-384 (cat # 6007290) Light gray AlphaPlate-384 (cat # 6005350)
	12 500	20 µL	2 µL	2 µL	16 µL	Light gray AlphaPlate-384 (cat # 6005350) ProxiPlate-384 Plus (cat # 6008280) White OptiPlate-384 (cat # 6007290)
	25 000	10 µL	1 µL	1 µL	8 µL	Light gray AlphaPlate-1536 (cat # 6004350)

High Sensitivity Protocol (2 incubation steps) 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:
Add 5 mL of 10X AlphaLISA Immunoassay Buffer to 45mL Milli-Q water.

2) Preparation of 1X AlphaLISA Lysis Buffer
Add 2 mL of 5X AlphaLISA Lysis Buffer to 8 mL Milli-Q water.

3) Preparation of BTK analyte standard dilutions:

a. Prepare standard dilutions as follows in 1X AlphaLISA Lysis Buffer (change tip between each standard dilution.) See p4 for storing remaining analyte:

Tube	Vol. of BTK (µL)	Vol. of diluent (µL) *	[BTK] in standard curve	
			(g/mL in 5 µL)	(pg/mL in 5 µL)
A	10 µL of provided BTK	90	3.00E-07	300 000
B	60 µL of tube A	120	1.00E-07	100 000
C	60 µL of tube B	140	3.00E-08	30 000
D	60 µL of tube C	120	1.00E-08	10 000
E	60 µL of tube D	140	3.00E-09	3 000
F	60 µL of tube E	120	1.00E-09	1 000
G	60 µL of tube F	140	3.00E-10	300
H	60 µL of tube G	120	1.00E-10	100
I	60 µL of tube H	140	3.00E-11	30
J	60 µL of tube I	120	1.00E-11	10
K	60 µL of tube J	140	3.00E-12	3
L	60 µL of tube K	120	1.00E-12	1
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 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. The remaining analyte should be stored at -80°C for further experiments.

** 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).

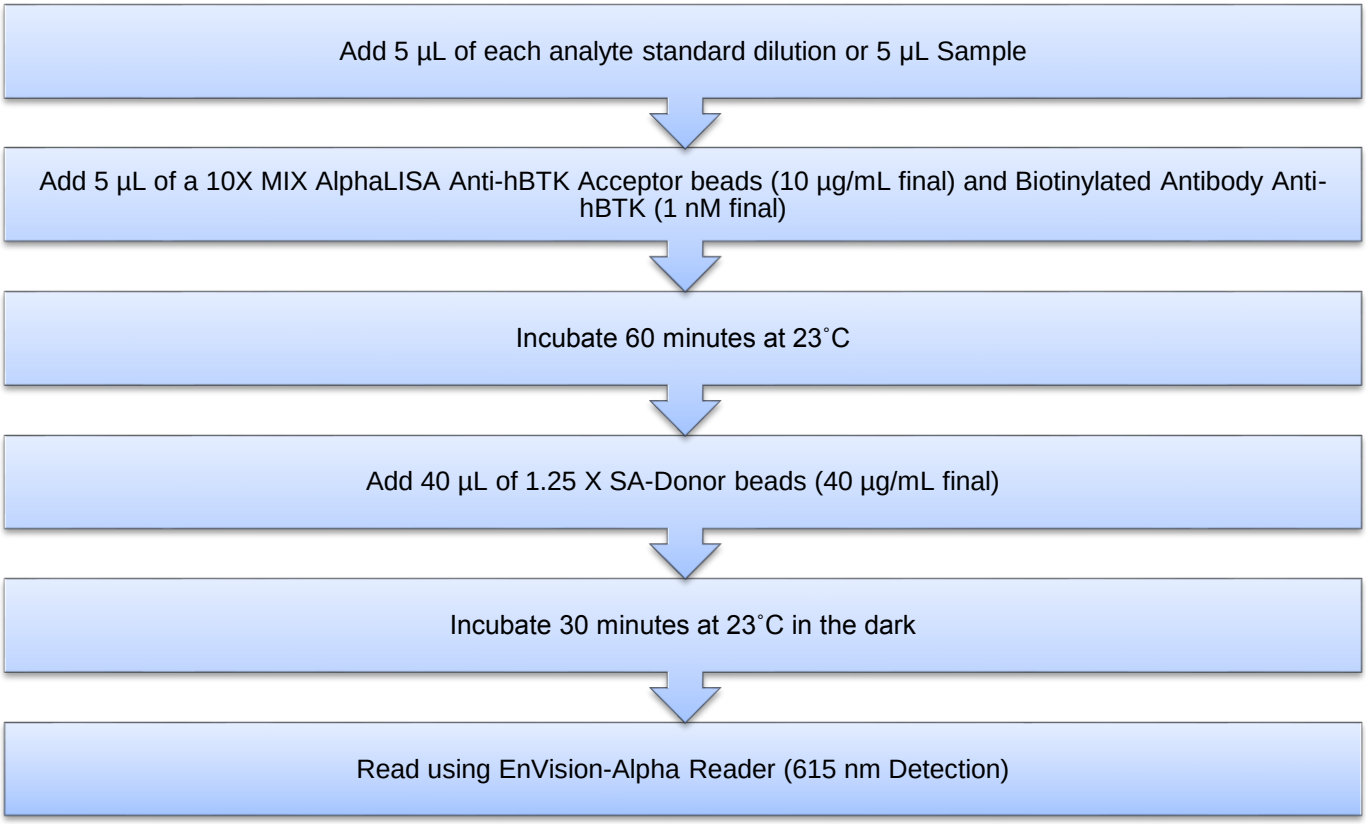
4) Preparation of 10X MIX AlphaLISA Anti-hBTK Acceptor beads (100 µg/mL) + Biotinylated Anti-hBTK Antibody (10 nM):

a. Prepare just before use.
b. Add 50 µL of 5 mg/mL AlphaLISA Anti-hBTK Acceptor beads and 50 µL of 500 nM biotinylated to 2400 µL of 1X AlphaLISA Immunoassay Buffer.

5) Preparation of 1.25X Streptavidin (SA) Donor beads (50 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 19800 µL of 1X AlphaLISA Immunoassay Buffer.

6) 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 high sensitivity protocol performed with standard diluted in AlphaLISA Lysis buffer and other reagents in AlphaLISA Immunoassay Buffer.

- Assay Sensitivity:

The LDL and LLOQ were 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 *	# of experiments
25.5	AlphaLISA Lysis Buffer	6

* The standard was prepared in this diluent. Note that LDL/ LLOQ can be decreased (i.e. sensitivity increased) by preparing standards in different matrixes.

- 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 AlphaLISA 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 IAB to dilute the beads and biotinylated antibodies.

- Intra-assay precision:

The intra-assay precision was determined using a total of 16 independent determinations in triplicate. Shown as CV%.

hBTK	AlphaLISA Lysis Buffer
CV(%)	5

- Inter-assay precision:

The inter-assay precision was determined using a total of 3 independent determinations with 9 measurements for 30 ng/mL sample. Shown as CV%.

hBTK (30 ng/ml)	AlphaLISA Lysis Buffer
CV (%)	14

- Spike Recovery:

Three known concentrations of analyte were spiked in AlphaLISA Lysis Buffer. All samples, including non-spiked buffer were measured in the assay. The average recovery from three independent measurements is reported. Note that the standard curves were prepared in Alpha Lysis Buffer.

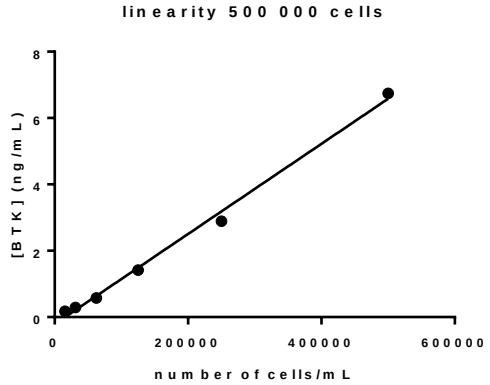
Spiked hBTK (ng/mL)	% Recovery
	AlphaLISA Lysis Buffer
30	95
10	89
3	97

Cell Lysate Experiments

Daudi cells (a B-cell line) were grown in RPMI1640 + 10% FBS in a 37°C incubator with 5% CO_2 and harvested at confluency. After counting, quantities of 500 000, 250 000, 125 000, 62 500, 31 250 and 16 625 cells were pipetted directly from the culture flask. The cells were washed twice with sterile PBS by centrifugation, and then 1 000 µL of Alpha Lysis Buffer was added to each quantity. The cells were incubated at 37°C for 30 minutes and the lysates were then tested.

5 µL of each lysate was tested in triplicate and a standard curve was performed using the above protocol were the analyte dilutions were performed in Alpha Lysis buffer. All other reagents were prepared in IAB. Results show good linearity between number of cells and amounts of protein detected (R^2 fit 0.9957).

Amount of cells	[BTK] (ng/mL)	Amount per cell (fg/mL)
500 000	6.74	13.4
250 000	2.89	11.6
125 000	1.42	11.4
62 500	0.58	9.28
31 250	0.29	9.28
15 625	0.18	11.5



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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