TECHNICAL DATA SHEET

AlphaLISA® Research Reagents

Caution: For Laboratory Use. A research chemical for research purposes only.

Residual Protein A Kit

Product No.: AL287 C/F

Lot specific kit information can be found at www.perkinelmer.com/COA

Material Provided

Format: AL287C: 500 assay points AL287F: 5 000 assay points
The number of assay points is based on an assay volume of 50 µL in 96- or 384-well assay plates using the kit components at the recommended concentrations.

Product Information

Kit content: The kit contains 6 components: AlphaLISA Acceptor beads coated with an Anti-Analyte Antibody, Streptavidin-coated Donor beads, Biotinylated Anti-Analyte Antibody, lyophilized analyte, 3X Dissociation Buffer and 10X AlphaLISA HiBlock Buffer.
The Phosphate / Tween buffer suggested to prepare the standards is not provided in the kit.

Assay microplates (96-, 384- or 1536-well plates) must be purchased separately (see page 3 for more details).

Storage: Store kit in the dark at +4˚C. Store reconstituted analyte at -20°C.

Stability: This product is stable for at least 12 months from the manufacturing date when stored in its original packaging and the recommended storage conditions. Note: Once reconstituted, the Protein A analyte is stable for at least 75 days at -20°C (see page 2: Reagents and Materials).

Application: This kit is designed for the quantitative determination of Protein A in buffered solution using a homogeneous AlphaLISA assay (no wash steps).

Sensitivity: Lower Detection Limit (LDL): 10.8 pg/mL (see page 9: Assay Performance Characteristics).

Dynamic range: 10.8 – 30 000 pg/mL (see page 9: Assay Performance Characteristics).

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Quality Control

Lot to lot consistency is confirmed in an AlphaLISA assay. Maximum and minimum signals, EC_{50} and LDL were measured on an EnVision® HTS instrument using the High sensitivity 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 depending on assay conditions with no impact on LDL measurement.

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Precautions

- Only the AlphaScreen® 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. Some analytes are from 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.

Reagents and Materials

The reagents provided in the AlphaLISA kit are listed in the table below:

Kit components	**AL287C (500 assay points)**	**AL287F (5 000 assay points)**
AlphaLISA Anti-Protein A Acceptor beads stored in PBS, 0.05% Proclin-300, pH 7.2	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	200 µL @ 5 mg/mL (1 brown tube, black cap)	2 X 1 mL @ 5 mg/mL (2 brown tubes, black caps)
Biotinylated Antibody Anti-Protein A stored in PBS, 0.1% Tween-20, 0.05% NaN_3, pH 7.4	50 µL @ 500 nM (1 tube, black cap)	500 µL @ 500 nM (1 tube, black cap)
AlphaLISA Protein A (0.3 µg), lyophilized analyte *	1 tube, clear cap	1 tube, clear cap
AlphaLISA Dissociation Buffer (3X) **	10 mL, 1 small bottle	100 mL, 1 large bottle
AlphaLISA HiBlock Buffer (10X) ***	10 mL, 1 small bottle	100 mL, 1 large bottle

* Reconstitute Protein A in 100 µL Milli-Q® grade H_2O. The reconstituted analyte should be used within 60 minutes, if possible, or aliquoted into screw-capped polypropylene vials and stored at -20°C for further experiments. Avoid multiple freeze-thaw cycles. It has been demonstrated that reconstituted Protein A is stable for at least 75 days at -20°C. One vial contains an amount of Protein A sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AL287S).

** The 3X Dissociation Buffer is not soluble when stored at 4°C. For 10 mL bottles, before use, warm up the buffer at 37ºC in a water bath for 5 minutes to re-dissolve. For 100 mL bottles, before use, warm up the buffer at 37ºC in a water bath for 30 minutes and shake gently to re-dissolve. The buffer contains 0.15% Proclin-300 as anti-microbial and 4.5% BSA, and other components that are not disclosed. The 3X buffer is stable for at least 2 months at room temperature. Extra buffer can be ordered separately (cat # AL006C: 10 mL, cat # AL006F: 100 mL).

*** Contains 250 mM HEPES, pH 7.4, 1% Casein, 10 mg/mL Dextran-500, 5% Triton X-100, 5% gelatin, 5% BSA and 0.5% Proclin-300. Extra buffer can be ordered separately (cat # AL004C: 10 mL, cat # AL004F: 100 mL).
Note: 10X buffer is slightly brown. However, this does not affect the assay results.

Once diluted, 1X AlphaLISA HiBlock Buffer contains 25 mM HEPES, pH 7.4, 0.1% Casein, 1 mg/mL Dextran-500, 0.5% Triton X-100, 0.5% gelatin, 0.5% BSA and 0.05% Proclin-300.

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).

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Specific additional required reagents and materials:

The following materials are recommended:

Item	Suggested source	Catalog #
TopSeal™-A Adhesive Sealing Film	PerkinElmer Inc.	6050195
EnVision®-Alpha Reader	PerkinElmer Inc.	-

Protocols have been optimized for 50 µL assays in white OptiPlate™-384 microplates. Other assay volumes can be used with similar protocols and identical final AlphaLISA reagent concentrations:

Format	# of data points	Total assay volume	Sample volume	AlphaLISA beads / Biotin Antibody MIX volume *	SA-Donor beads volume *	Plate recommendation
AL287C	250	100 µL	10 µL	40 µL	50 µL	White OptiPlate-96 (cat # 6005290)
	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)
AL287F	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)

* Volumes based on the Quick protocol.

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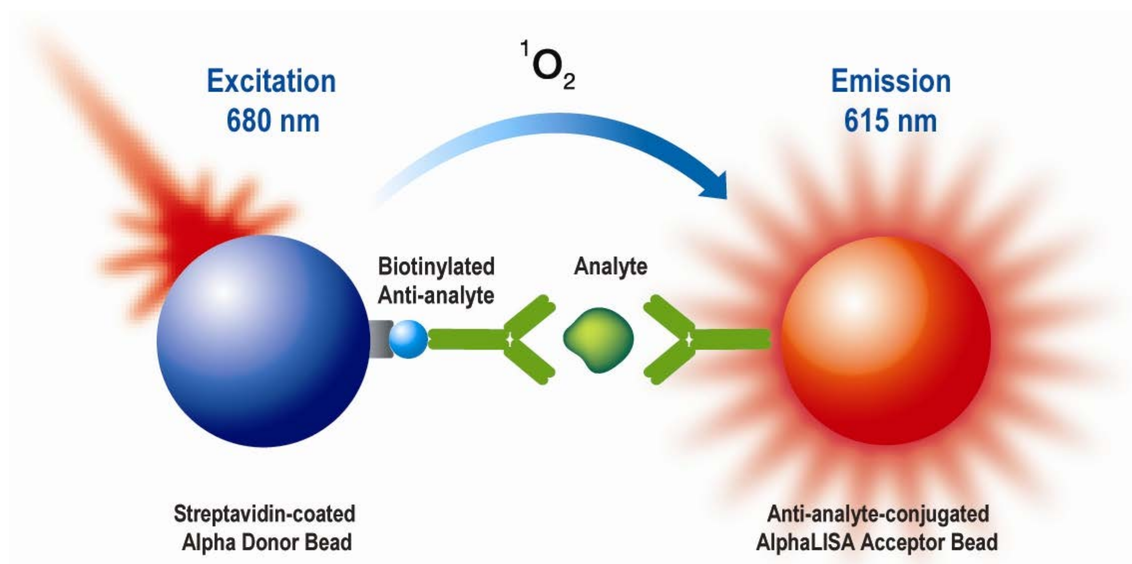
Analyte of Interest

Protein A is a 42 kDa protein widely used in affinity chromatography to purify antibodies, most importantly antibody therapeutics. The native form of Protein A from *S. Aureus*, in addition to many modified recombinant Protein A variants, are used in chromatography. The strong affinity and the high selectivity of Protein A for antibodies lead to an effective removal of host cell proteins. However, Protein A can be leached from columns to contaminate the antibody preparation. Since Protein A could elicit mitogenic or immunological reactions, there are strict regulations as to the acceptable levels of Protein A in antibody preparations. Therefore, there is a current need to quantitate Protein A in antibody preparations with excellent sensitivity and robustness, while maintaining high throughput capabilities.

Description of the AlphaLISA Assay

AlphaLISA technology allows the detection of molecules of interest in buffer, cell culture media, serum and plasma in a highly sensitive, quantitative, reproducible and user-friendly mode. In an AlphaLISA assay, a Biotinylated Anti-Analyte Antibody binds to the Streptavidin-coated Donor beads while another Anti-Analyte 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 (see figure below).



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Recommendations

General 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 prewet the tip.

- Centrifuge all tubes (including lyophilized analyte) before use to improve recovery of content (2 000 g, 10-15 sec). Resuspend all reagents by vortexing before use.

- Use Milli-Q® grade H_2O (18 MΩ•cm) to dilute the 10X AlphaLISA HiBlock 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 in 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 a similar matrix as the samples (e.g. FBS for serum samples).

Specific recommendations:

- The standard curve can be diluted in the same matrix as the samples. Refer to recovery table on page 10. The addition of detergent, such as 0.1% Tween, to the standard diluent prevents the loss of Protein A due to non-specific binding.

- It is suggested to use a Polymerase Chain Reaction (PCR) instrument for heating of standards and samples.

Protocols

The two protocols described below are 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. 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 is calculated. One background point in triplicate (3 wells) can be used when LDL is not calculated.

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Protocol 1: Quick protocol (2 incubation steps) – Dilution of standards in Phosphate / Tween buffer

Protocol 2: High sensitivity protocol (3 incubation steps) – Dilution of standards in Phosphate / Tween buffer

IMPORTANT: PLEASE READ THE RECOMMENDATIONS ABOVE BEFORE USE

Common Steps for Preparing Reagents (Protocols 1 & 2)

If a different amount of samples are tested, the volumes of all reagents have to be adjusted accordingly.

1) Preparation of Phosphate / Tween buffer:
Add 250 µL of 10X Phosphate Buffered Saline (PBS), 250 µL of 5M NaCl, 25 µL of Tween 10% to 1 975 µL H_2O. 10X PBS contains 10.6 mM Potassium Phosphate monobasic (KH_2PO_4), 1 552 mM Sodium Chloride (NaCl) and 29.7 mM Sodium Phosphate dibasic (Na_2HPO_4-$7H_2O$).

2) Preparation of Protein A analyte standard dilutions:
Reconstitute lyophilized Protein A (0.3 µg) in 100 µL H_2O.
Prepare standard dilutions as follows in (change tip between each standard dilution):

Tube	Vol. of Protein A (µL)	Vol. of diluent (µL) *	[Protein A] in diluent *	Final [Protein A] in standard curve	
			(g/mL)	(g/mL in 5 µL)	(pg/mL in 5 µL)
A	10 µL of reconstituted Protein A	190	1.50E-07	1E-07	100 000
B	60 µL of tube A	140	4.50E-08	3E-08	30 000
C	60 µL of tube B	120	1.50E-08	1E-08	10 000
D	60 µL of tube C	140	4.50E-09	3E-09	3 000
E	60 µL of tube D	120	1.50E-09	1E-09	1 000
F	60 µL of tube E	140	4.50E-10	3E-10	300
G	60 µL of tube F	120	1.50E-10	1E-10	100
H	60 µL of tube G	140	4.50E-11	3E-11	30
I	60 µL of tube H	120	1.50E-11	1E-11	10
J	60 µL of tube I	140	4.50E-12	3E-12	3
K	60 µL of tube J	120	1.50E-12	1E-12	1
L	60 µL of tube K	140	4.50E-13	3E-13	0.3
M ** (background)	0	100	0	0	0
N ** (background)	0	100	0	0	0
O ** (background)	0	100	0	0	0
P ** (background)	0	100	0	0	0

* Dilute standards in diluent (e.g. Phosphate / Tween 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 standard curve and samples:
Dilute standards and samples in 3X Dissociation Buffer, for example, 10 µL of 3X Dissociation Buffer plus 20 µL of standards / samples. Then, heat standards and samples for 60 minutes at 98ºC. Centrifuge for 5 minutes at ≥ 200 g. Note that samples should not contain more than 1 mg/mL of IgG.

4) Preparation of 1X AlphaLISA HiBlock Buffer:
Add 2.5 mL of 10X AlphaLISA HiBlock Buffer to 22.5 mL H_2O.

Protocol 1: Quick Protocol (2 Incubation Steps)

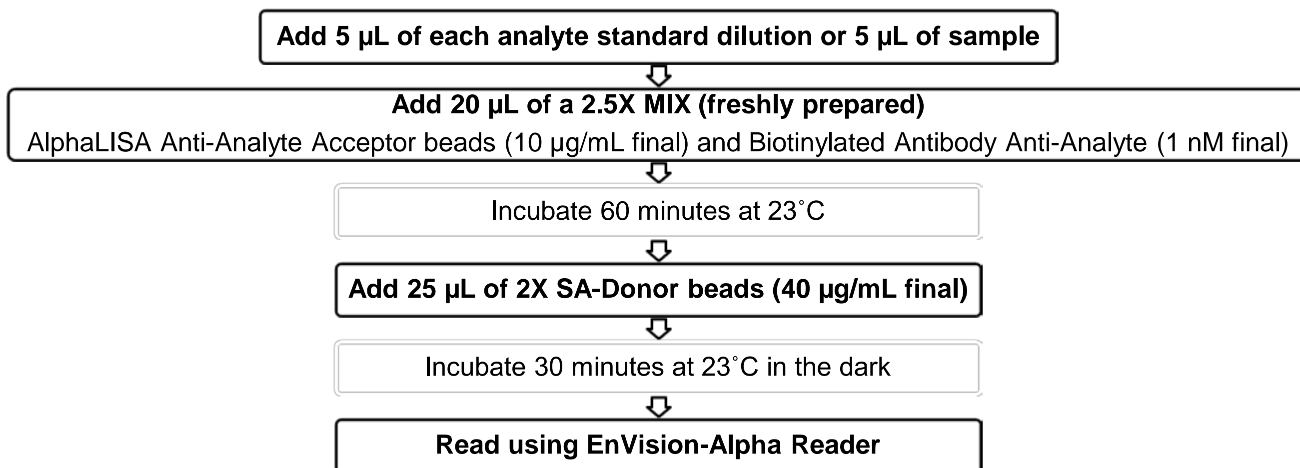
The protocol described below is for one standard curve (48 wells) and samples (452 wells). Dilution of standards can be done in Phosphate / Tween buffer.

If a different amount of samples are tested, the volumes of all reagents have to be adjusted accordingly.

5) Preparation of 2.5X AlphaLISA Anti-Protein A Acceptor beads + Biotinylated Antibody Anti-Protein A MIX (25 µg/mL / 2.5 nM):
Add 50 µL of 5 mg/mL AlphaLISA Anti-Protein A Acceptor beads and 50 µL of 500 nM Biotinylated Antibody Anti-Protein A to 9 900 µL of 1X AlphaLISA HiBlock Buffer. Prepare just before use.

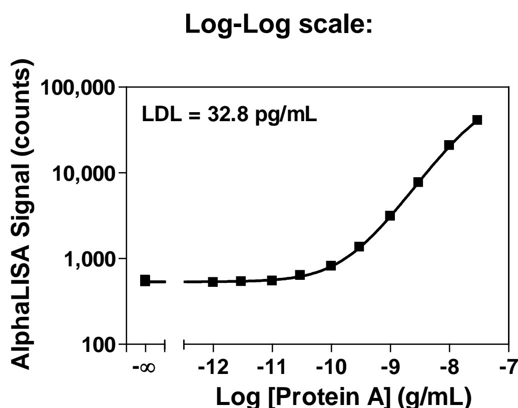
6) Preparation of 2X Streptavidin (SA) Donor beads (80 µg/mL): Keep the beads under subdued laboratory lighting. Add 200 µL of 5 mg/mL SA-Donor beads to 12 300 µL of 1X AlphaLISA HiBlock Buffer.

7) In a 96- or 384-well microplate:

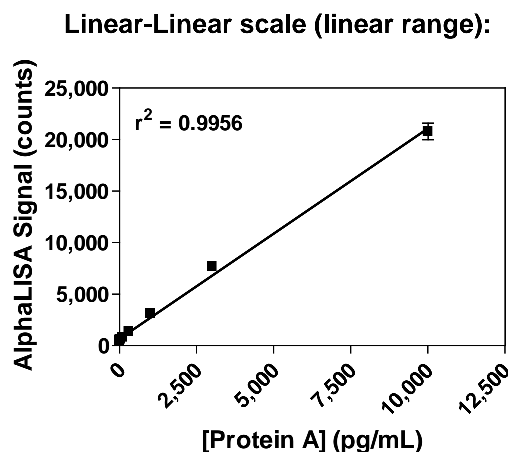


Protocol 1 - Typical results in Phosphate / Tween buffer

Log-Log scale:



Linear-Linear scale (linear range):



The data was generated using a white Optiplate-384 microplate and an EnVision-Alpha Reader 2102.

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Protocol 2: High Sensitivity Protocol (3 Incubation Steps)

The protocol described below is for one standard curve (48 wells) and samples (452 wells). Dilution of standards can be done in Phosphate / Tween buffer.

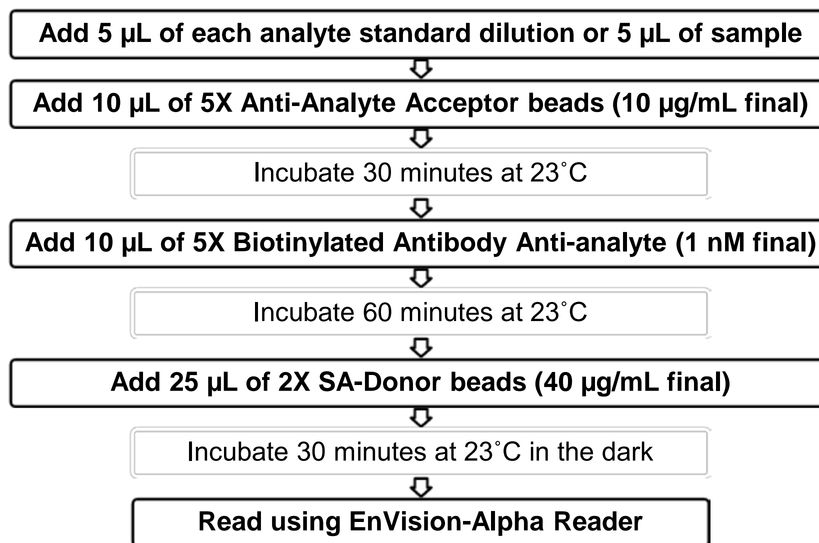
If a different amount of samples are tested, the volumes of all reagents have to be adjusted accordingly.

5) Preparation of 5X AlphaLISA Anti-Protein A Acceptor beads (50 µg/mL):
Add 50 µL of 5 mg/mL AlphaLISA Anti-Protein A Acceptor beads to 4 950 µL of 1X AlphaLISA HiBlock Buffer.

6) Preparation of 5X Biotinylated Antibody Anti-Protein A (5 nM):
Add 50 µL of 500 nM Biotinylated Antibody Anti-Protein A to 4 950 µL of 1X AlphaLISA HiBlock Buffer.

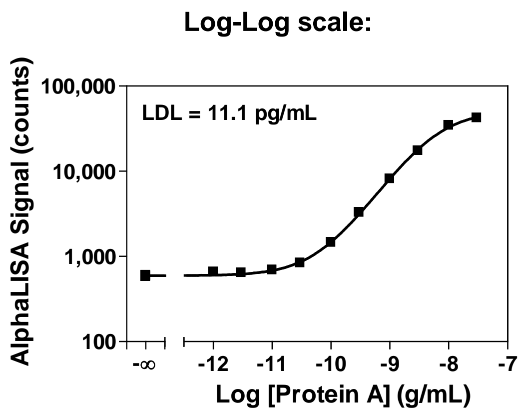
7) Preparation of 2X Streptavidin (SA) Donor beads (80 µg/mL): Keep the beads under subdued laboratory lighting.
Add 200 µL of 5 mg/mL SA-Donor beads to 12 300 µL of 1X AlphaLISA HiBlock Buffer.

8) In a 96- or 384-well microplate:

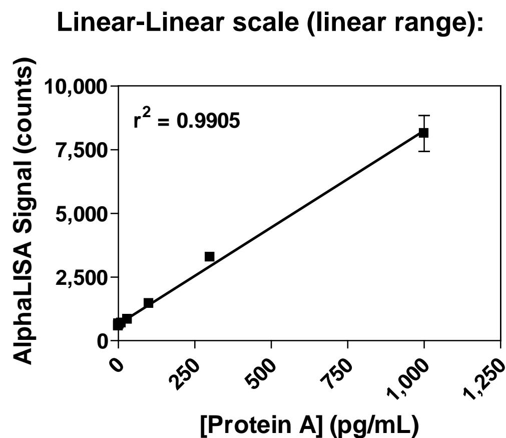


Protocol 2 - Typical results in Phosphate / Tween buffer

Log-Log scale:



Linear-Linear scale (linear range):



The data was generated using a white Optiplate-384 microplate and an EnVision-Alpha Reader 2102.

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Protocols 1 & 2 - Interpreting the Data

- 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.
- 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 High sensitivity protocol.

Sensitivity:

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

- Average LDL is 10.8 pg/mL * (using 5 µL of analyte) (mean of 18 independent experiments).

* Note that LDL can be decreased (i.e. sensitivity increased) by increasing the volume of analyte in the assay (e.g. use 10 µL of analyte in a final assay volume of 50 µL).

Dynamic range: 10.8 – 30 000 pg/mL

Assay precision:

The following assay precision data were calculated from a total of 18 assays. Two operators performed three independent assays using three different kit lots. Each assay consisted of one standard curve and three control samples of high (A), medium (B) and low (C) concentration, assayed in triplicate. The assays were performed in 384-well format.

- Intra-assay precision:

The intra-assay precision was determined using a total of 18 independent determinations in triplicate for each control sample.

Sample	Mean (pg/mL)	SD (pg/mL)	% CV (n = 18)
A	3 392	355	10.5
B	318	15.9	5.0
C	37	4.4	12.0

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- Inter-assay precision:

The inter-assay precision was determined using a total of 6 independent determinations with 9 measurements for each control sample.

Sample	Mean (pg/mL)	SD (pg/mL)	% CV (n = 6)
A	3 392	418	12.3
B	318	21.8	6.9
C	37	5.0	13.6

Recovery experiments:

In the following experiment, the recovery of spiked analyte was tested using five commonly used bioprocess buffers. All samples, including non-spiked buffer, were measured in the assay. Values calculated for spiked samples reflect subtraction of the non-spiked buffer. The % in buffer versus expected (control spike value) was calculated for each concentration. The average recovery from two independent measurements is reported.

Buffer	Spike (ng/mL)	% Recovery
PBS 0.5X, 0.5 M NaCl	3.0	88
	0.3	86
	0.03	79
Tris 50 mM, pH 8.0	3.0	91
	0.3	92
	0.03	96
Citrate / Phosphate pH 6.0	3.0	116
	0.3	94
	0.03	102
Phosphate buffer 50 mM, pH 6.0	3.0	92
	0.3	92
	0.03	104
Acetate buffer, pH 5.0	3.0	110
	0.3	99
	0.03	102

Specificity:

Cross-reactivity of the AlphaLISA Protein A Kit was tested using the following proteins at 10 ng/mL.

Protein	% Cross-reactivity
MabSelect SuRe™	71
MabSelect™ & MabSelect Xtra™	82

The possible interference from human IgG was investigated. The Protein A was kept at a constant concentration (EC_{50} value of the standard curve). The binding protein was titrated into the assay. No interference was observed up to 1 mg/mL.

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