AlphaLISA® Cortisol Kit

Product number: AL314 HV/C/F

Research Use Only. Not for use in diagnostic procedures.

Product Information

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Application:	This kit is designed for the quantitative determination of cortisol, using a homogeneous AlphaLISA assay (no wash steps). The assay shows negligible cross-reactivity with other steroids of similar structure.
Sensitivity:	Lower Detection Limit (LDL): 177 pg/mL
	Lower Limit of Quantification (LLOQ): 1242 pg/mL
	EC_{50}: 5.2 ng/mL
Dynamic range:	1000 - 3 600 000 pg/mL (Figure 1).
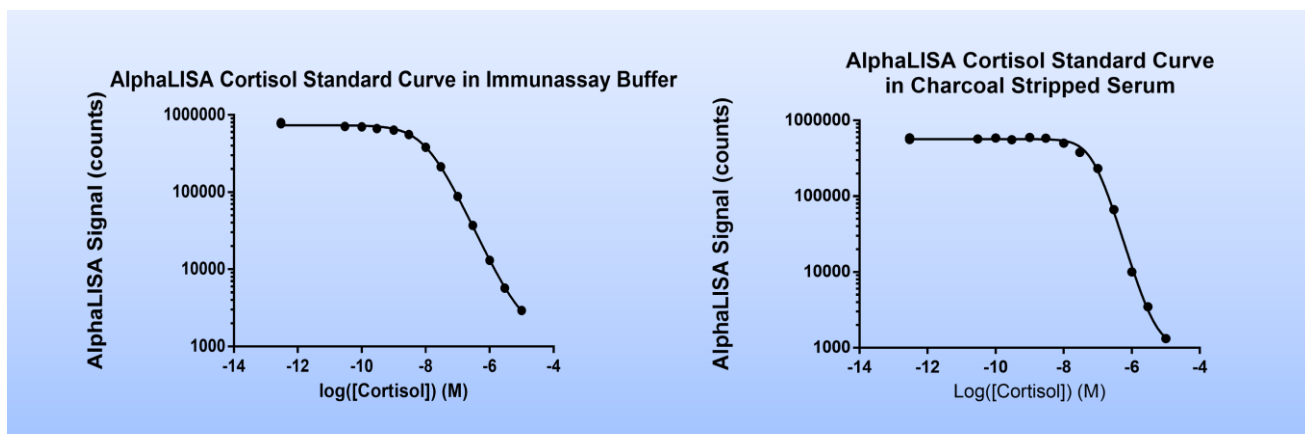
Figure. 1. Typical sensitivity curves in AlphaLisa buffer (left) and serum (right). The data was generated using a white Optiplate™-384 microplate and the EnVision® Multilabel Plate Reader with Alpha option 2102.

Storage:	Store kit in the dark at +4˚C. Store reconstituted analyte at -20°C.
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. Note: Once reconstituted, the anti-Cortisol Antibody is stable for at least 6 months when stored at -20°C.

Analyte of Interest

Cortisol (hydrocortisone) is a steroid derived from cholesterol. Cortisol can be found in the blood as free or bound to cortisol-binding globulins, such as transcortin. Cortisol levels follow a diurnal pattern, typically highest in the morning upon wake up and lowest just before sleep. Cortisol levels have been linked to metabolism regulation, glucogenesis promotion, liver glycogen deposition, and depression of glucose burn. Cortisol levels have also been positively correlated to stress and increasing blood pressure.

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 competition assay, a Biotinylated analog of the analyte of interest, the tracer, binds to the Streptavidin-coated Alpha Donor beads, while the Anti-Analyte Antibody is conjugated to AlphaLISA Acceptor beads. In the presence of low 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). In the presence of high analyte, the beads are separated resulting in lower emission.
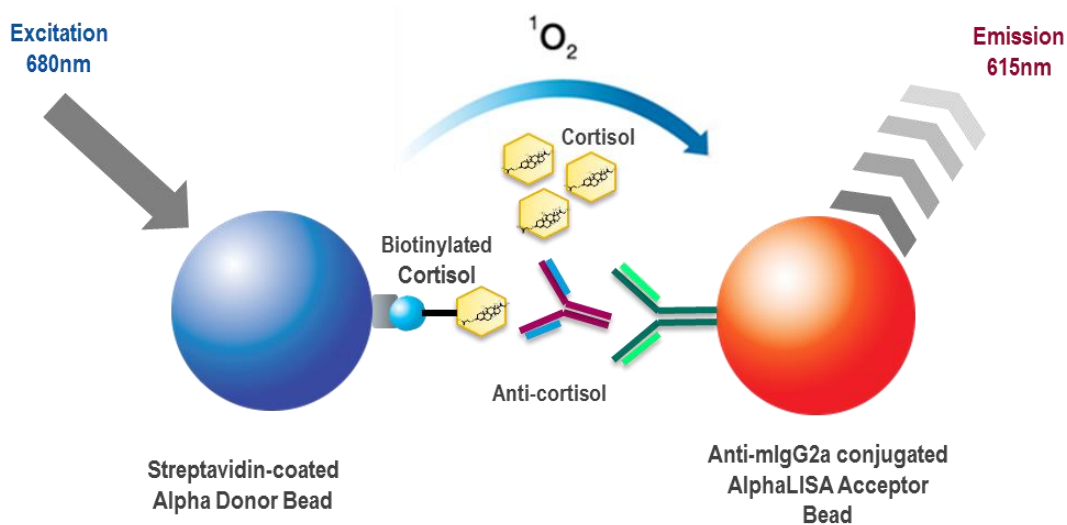

Figure 2. AlphaLISA Assay principle.

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.
- Some analytes are present in saliva. Take precautionary measures to avoid contamination of the reagent solutions.

Kit Content: Reagents and Materials

Kit components	AL314HV (100 assay points***)	AL314C (500 assay points***)	AL314F (5 000 assay points***)
AlphaLISA Acceptor beads stored in PBS, 0.05% Kathon, pH 7.2	40 µL @ 5 mg/mL (1 brown tube, <u>white</u> cap)	100 µL @ 5 mg/mL (1 brown tube, <u>white</u> cap)	1000 µL @ 5 mg/mL (1 brown tube, <u>white</u> cap)
Streptavidin (SA)-coated Donor beads stored in 25 mM HEPES, 100 mM NaCl, 0.05% Kathon, pH 7.4	40 µL @ 5 mg/mL (1 brown tube, <u>black</u> cap)	100 µL @ 5 mg/mL (1 brown tube, <u>black</u> cap)	1000 µL @ 5 mg/mL (1 brown tube, <u>black</u> caps)
Anti-Cortisol Antibody, 0.4 ug	1 clear tube, black cap	1 tube, black cap	10 tubes, black cap
Biotinylated Cortisol-Tracer 100X Stored in 1X PBS, 10% DMSO	100 µL @ 5 µM (1 tube, <u>black</u> cap)	250 µL @ 5 µM (1 tube, <u>black</u> cap)	2500 µL @ 5 µM (2 tubes, <u>black</u> cap)
Cortisol Analyte Standard Stored in 1X PBS, 50% DMSO	100 µL @ 100 µM 1 tube, <u>clear</u> cap	100 µL @ 100 µM 1 tube, <u>clear</u> cap	100 µL @ 100 µM 1 tube, <u>clear</u> cap
AlphaLISA ImmunoAssay Buffer (10X) **	2 mL, 1 small bottle	10 mL, 1 small bottle	100 mL, 1 large bottle

*   Reconstitute anti-Cortisol Antibody in 500 µL Milli-Q® grade H_2O. The reconstituted analyte should be used within 60 minutes 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 anti-Cortisol Antibody is stable for at least 6 months at -20°C. One vial contains an amount of anti-Cortisol Antibody sufficient for performing 10 standard curves.

**   Extra buffer can be ordered separately (cat # AL000C: 10 mL, cat # AL000F: 100 mL).

***   The number of assay points is based on an assay volume of 100 µL in 96-well plates (AL314HV) or 50 µL in 96- or 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 Adhesive Sealing Film	Revvity Inc.	6050185
EnVision®-Alpha Reader	Revvity 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$_2$O (18 MΩ•cm) to dilute 10X AlphaLISA ImmunoAssay Buffer 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 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).

- AlphaLISA assays can be performed in cell culture medium with or without phenol red, with the following recommendations: if possible, avoid biotin-containing medium (e.g. RPMI medium) as lower counts and lower sensitivity are expected. Add at least 1% FBS or 0.1% BSA to cell culture medium.

Assay Procedure

IMPORTANT: PLEASE READ THE RECOMMENDATIONS BELOW BEFORE USE

- The manual described below is an example for generating one standard curve in a 50 µL final assay volume (48 wells, triplicate determinations). The manuals 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 manual is provided for information only. As needed, the number of replicates or the range of concentrations covered can be modified.

- Use of four MAX points in triplicate (12 wells) is recommended when LDL/LLOQ is calculated. One MAX point in triplicate (3 wells) can be used when LDL/LLOQ is not calculated

- Use of null (MIN) points in triplicate (12 wells) is recommended when normalizing data. Analyzing true signal data does not require null points

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

Standard manual (3 incubation steps) – Dilution of standards in 1X AlphaLISA ImmunoAssay Buffer or Charcoal Stripped Serum

The manual described below is recommended when generating one standard curve in a 50 µL final assay volume (48 wells, triplicate determinations with manual pipetting). *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 2 mL of 10X AlphaLISA ImmunoAssay Buffer to 18 mL H_2O.

2) Preparation of Cortisol analyte standard dilutions:

   • Dilute Cortisol analyte standard (100 µM) in AlphaLISA Immunoassay Buffer. The first point of the curve is 10 µM so a 10 fold dilution of the provided solution is required.

   • Prepare standard dilutions as follows (change tip between each standard dilution) in 1X AlphaLISA ImmunoAssay Buffer or cell culture medium:

Tube	Vol. of Cortisol (µL)	Vol. of diluent (µL) *	[Cortisol] in standard curve	
			(µM in 20 µL)	(pg/mL in 20 µL)
A	20 µL of Cortisol std	180	10	3623188
B	60 µL of tube A	140	3	1086956
C	60 µL of tube B	120	1	362318
D	60 µL of tube C	140	0.3	108695
E	60 µL of tube D	120	0.1	36231
F	60 µL of tube E	140	0.03	10869
G	60 µL of tube F	120	0.01	3623
H	60 µL of tube G	140	0.003	1086
I	60 µL of tube H	120	0.001	362
J	60 µL of tube I	140	0.0003	108
K	60 µL of tube J	120	0.0001	36
L	60 µL of tube K	140	0.00003	10
M ** (MAX)	0	100	0	0
N ** (MAX)	0	100	0	0
O ** (MAX)	0	100	0	0
P ** (MAX)	0	100	0	0
Q *** (null)	0	100	0	0
R *** (null)	0	100	0	0
S *** (null)	0	100	0	0
T*** (null)	0	100	0	0

*   Dilute standards in diluent (e.g. 1X AlphaLISA ImmunoAssay Buffer, cell culture medium, charcoal stripped serum).
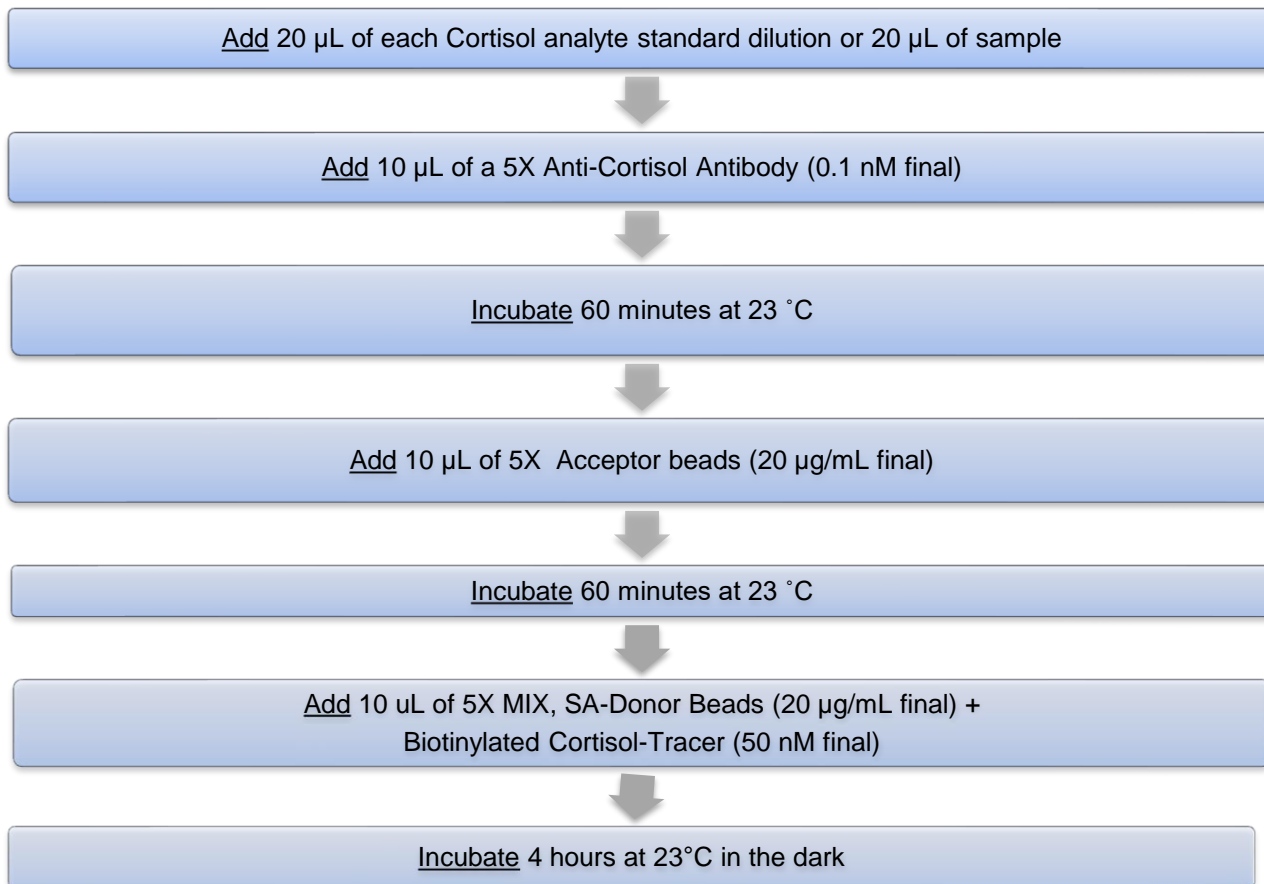   At low concentrations of analyte, a significant amount of analyte can bind to the vial. Therefore, load the analyte standard dilutions in the assay microplate within 60 minutes of preparation.
**   Four background points in triplicate (12 wells) are used when LDL/LLOQ is calculated. These represent the MAX signal in the assay. If LDL/LLOQ does not need to be calculated, one background point in triplicate can be used (3 wells).
*** Four null points in triplicate (12 wells) are used to determine the MIN signal of the assay (lowest possible counts). These wells contain anti-Cortisol Acceptor Bead, Anti-Cortisol Antibody, and SA-Donor Bead, and Buffer.

3) Preparation of 5X Anti-Cortisol Antibody (0.5 nM):
    a. Dissolve 0.4 µg (2.5 pmol) of lyophilized antibody in 500 µL Milli-Q water.
    b. Add 50 µL of Antibody to 450 µL of 1X Immunoassay Buffer
    c. Prepare just before use
4) Preparation of 5X AlphaLISA Anti-Cortisol Acceptor beads (100 µg/mL):
    a. Add 10 µL of 5 mg/mL AlphaLISA Anti-Cortisol Acceptor beads to 490 µL of 1X AlphaLISA ImmunoAssay Buffer.
    b. Prepare just before use

5) Preparation of **5X MIX** of Streptavidin (SA) Donor beads (100 µg/mL) + Biotinylated Cortisol-Tracer (250 nM):
    a. Keep Donor beads under subdued laboratory lighting!
    b. Add 10 µL of 5 mg/mL SA-Donor beads and 25 µL of 100X Biotinylated Cortisol-Tracer to 465 µL of 1X AlphaLISA ImmunoAssay Buffer.

6) Preparation of 5X Streptavidin (SA) Donor beads (100 µg/mL) **(For null points)**:
    a. Keep the beads under subdued laboratory lighting!
    b. Add 10 µL of 5 mg/mL SA-Donor beads to 490 µL of 1X AlphaLISA ImmunoAssay Buffer.

7) Samples:

    • If applicable, dilute samples to be tested in diluent (e.g. 1X AlphaLISA ImmunoAssay Buffer).

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

Add 20 µL of each Cortisol analyte standard dilution or 20 µL of sample

↓

Add 10 µL of a 5X Anti-Cortisol Antibody (0.1 nM final)

↓

Incubate 60 minutes at 23 ˚C

↓

Add 10 µL of 5X Acceptor beads (20 µg/mL final)

↓

Incubate 60 minutes at 23 ˚C

↓

Add 10 uL of 5X MIX, SA-Donor Beads (20 µg/mL final) +
Biotinylated Cortisol-Tracer (50 nM final)

↓

Incubate 4 hours at 23°C in the dark

Data Analysis (Direct)

- 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 MAX counts (12 wells without analyte) - 3 x standard deviation value (average MAX counts - (3xSD)) on the standard curve.
- The LLOQ as measured here is calculated by interpolating the average MAX 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.

Data Analysis (Normalized)

- The Cortisol AlphaLISA Competition Assay is strongly dependent on tracer concentration. Small changes in concentration can strongly affect top and bottom counts.
- It is recommended to convert data to normalized response. This requires a set of "null points" These wells contain only AlphaLISA anti-Cortisol Acceptor Bead, Anti-Cortisol Antibody, AlphaLISA SA-Donor Bead and buffer made up to a volume consistent with the standard curve.
- Calculate the average count value for the "null points" (12 wells without analyte or tracer).
- Generate a standard curve by plotting the AlphaLISA counts versus the concentration of analyte. A log scale can be used for the X-axis
- Normalize the data using the following equation (1-((sample counts – Avg (null points))/ Avg (MAX)))*100
- Analyze data according to a nonlinear regression using the 4-parameter logistic equation (sigmoidal normalized dose-response curve with variable slope) **Do not weight this data**
- Data will be presented as % inhibition (percentage of tracer inhibited by analyte).
- The LDL is calculated by interpolating the average normalized MAX counts (12 wells without analyte) + 3 x standard deviation value (average normalized MAX counts + (3xSD)) on the standard curve.
- The LLOQ as measured here is calculated by interpolating the average normalized MAX counts (12 wells without analyte) - 10 x standard deviation value (average MAX 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.

Normalized AlphaLISA Cortisol Standard Curve in Immunoassay Buffer
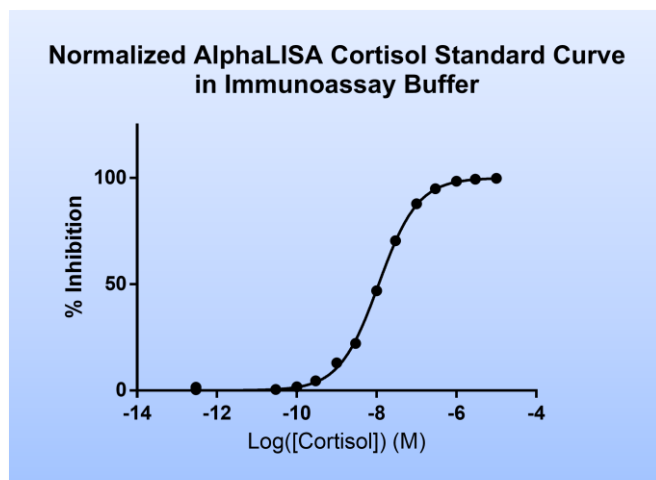
Assay Performance Characteristics

AlphaLISA assay performance described below was determined using the quick manual.

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 20 µL using the recommended assay conditions.

LDL	LLOQ (ng/ml)	Buffer/Media used	# of experiments
177 pg/ml	1.24	AlphaLisa ImmunoAssay Buffer	10
6.69 ng/ml	29.21	Charcoal stripped Serum	3

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 ImmunoAssay Buffer (IAB) and charcoal stripped serum. 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 AlphaLISA ImmunoAssay Buffer.

- Intra-assay precision:

The intra-assay precision was determined using a total of 10 independent determinations in triplicate. Shown CV%.

Cortisol (pg/ml)	IAB	Serum
3600	3%	4%

- Inter-assay precision:

The inter-assay precision was determined using a total of 6 independent determinations. Shown CV%.

| Cortisol (pg/ml)	Buffer	Serum
3600	4%	9%

- Specificity:

Cross-reactivity of the AlphaLISA Cortisol Kit was tested using the following steroids at 36 ng/mL in AlphaLISA ImmunoAssay Buffer:

Steroid	% Cross-reactivity
Prednisolone	5%
17a-hydroxyprogesterone	0%
Progesterone	0%
Cortisone	1%
Corticosterone	1%
21-deoxycortisol	9%

Troubleshooting Guide

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

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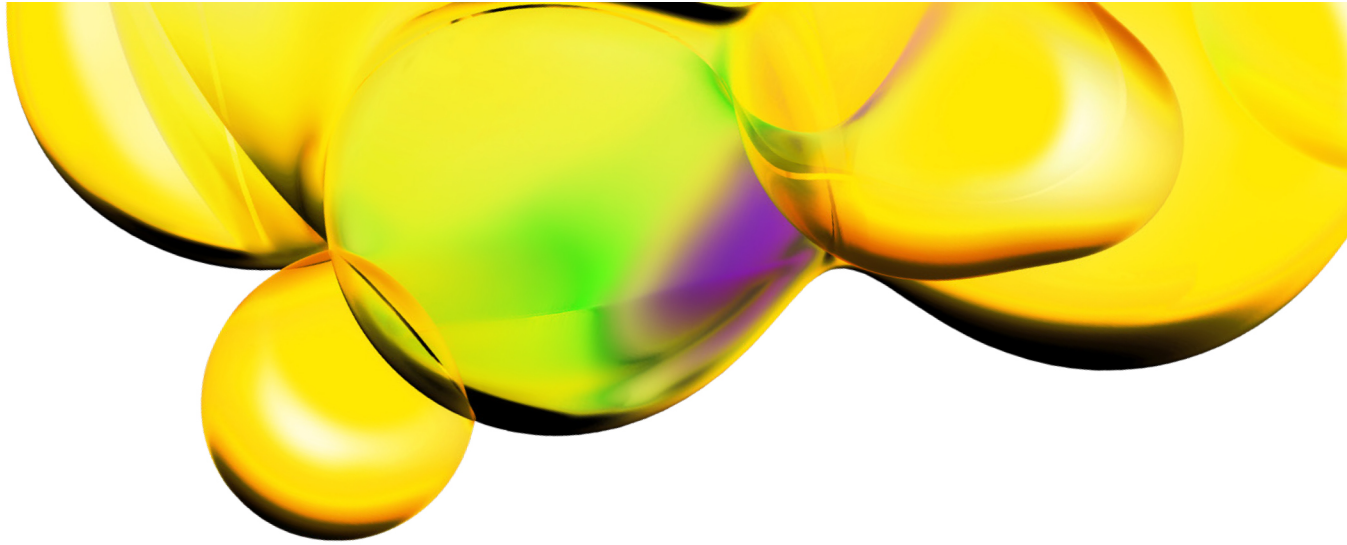
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