TECHNICAL DATA SHEET

AlphaLISA® Research Reagents

AlphaLISA Testosterone Detection Kit

Product number: AL324 HV/C/F

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

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

Contents

Product Information

Application: This kit is designed for the quantitative determination of testosterone, using a homogeneous AlphaLISA assay (no wash steps). The assay shows negligible cross-reactivity with other steroids of similar structure.

Sensitivity: Broad Range Assay:

Lower Detection Limit (LDL): 0.7 nM

High Sensitivity Assay:

Lower Detection Limit (LDL): 0.035 nM

Dynamic range: Broad Range Assay: 0.7 - 875 nM (Figure 1).

High Sensitivity Assay: 0.035 – 8.5 nM

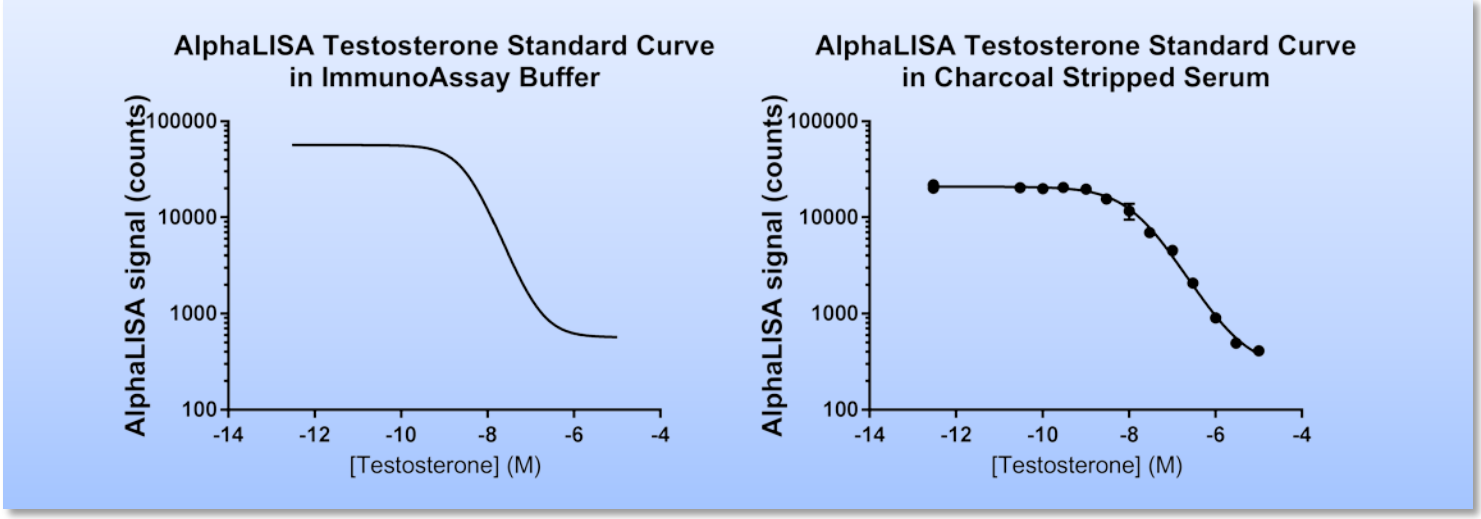


Figure. 1. Typical sensitivity curves in AlphaLISA buffer (left) and serum (right). The data was generated using a white Optiplate™-384 microplate and the EnVision® Multilabel Plate Reader 2103 with Alpha option.

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

Stability: This 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

Testosterone is the predominant androgenic steroid. This steroid hormone has many functions both in reproductive and non-reproductive tissue. Testosterone plays a key role in the development of testis and prostate. Testosterone levels are also linked with muscle, bone, and body hair growth. Testosterone is observed in most verterbrates, with fish being the largest exception. Testosterone has been used as a treatment for hypogonadism, infertility, osteoporosis, anemia, and low appetite

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.

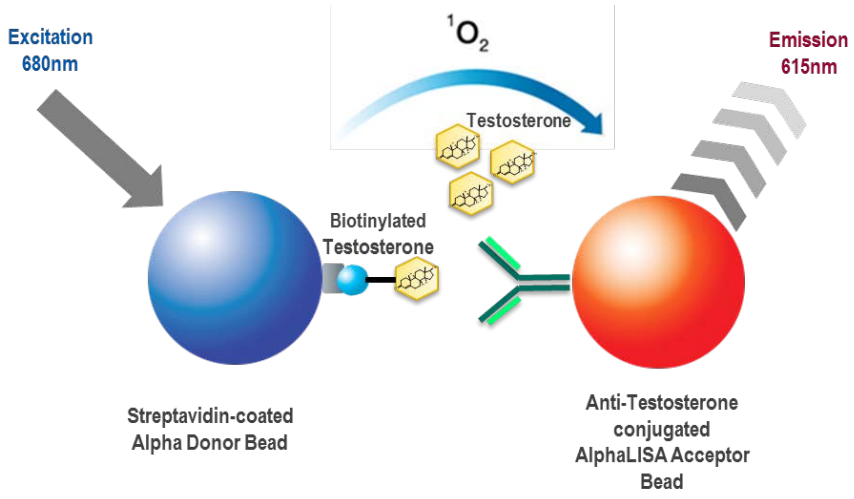


Figure 2. AlphaLISA Assay principle.

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	AL 324HV (100 assay points*)	AL 324C (500 assay points*)	AL 324F (5 000 assay points*)
AlphaLISA Acceptor beads stored in PBS, 0.05% Proclin-300, pH 7.2	40 µL @ 5 mg/mL (1 brown tube, white cap)	100 µL @ 5 mg/mL (1 brown tube, white cap)	1000 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	40 µL @ 5 mg/mL (1 brown tube, black cap)	100 µL @ 5 mg/mL (1 brown tube, black cap)	1000 µL @ 5 mg/mL (1 brown tube, black caps)
Biotinylated Testosterone-Tracer Stored in 100% DMSO	10 µL @ 100 µM (1 tube, black cap)	10 µL @ 100 µM (1 tube, black cap)	50 µL @ 100 µM (1 tube, black cap)
Testosterone Analyte Standard Stored in 100% DMSO	200 µL @ 100 µM 1 tube, clear cap	200 µL @ 100 µM 1 tube, clear cap	200 µL @ 100 µM 1 tube, clear cap
AlphaLISA ImmunoAssay Buffer (10X) **	2 mL, 1 small bottle	10 mL, 1 small bottle	100 mL, 1 large bottle

* The number of assay points is based on an assay volume of 100 µL in 96-well plates (AL324HV) or 50 µL in 96- or 384-well assay plates using the kit components at the recommended concentrations.

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

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	PerkinElmer Inc.	6050195
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 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. charcoal-stripped serum 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. This specific assay is incompatible with RPMI.

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 MAX points in triplicate (12 wells) is recommended when LDL is calculated. One MAX point in triplicate (3 wells) can be used when LDL 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

		Volume				
Format	**# of data points**	**Final**	**Sample**	**AlphaLISA Acceptor Bead**	**SA-Donor beads /Tracer MIX**	**Plate recommendation**
324HV	100	100 µL	40 µL	20 µL	20 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
324C	250	100 µL	40 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	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	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	Light gray AlphaPlate-1536 (cat # 6004350)
324F	5 000	50 µL	20 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	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	Light gray AlphaPlate-1536 (cat # 6004350)

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

The protocol 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 Testosterone analyte standard dilutions:
   - Dilute Testosterone 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 Testosterone (µL)	Vol. of diluent (µL) *	[Testosterone] in standard curve	
			(µM in 20 µL)	(pg/mL in 20 µL)
A	20 µL of Testosterone std	180	10	2857142
B	60 µL of tube A	140	3	857142
C	60 µL of tube B	120	1	285714
D	60 µL of tube C	140	0.3	85714
E	60 µL of tube D	120	0.1	28571
F	60 µL of tube E	140	0.03	8571
G	60 µL of tube F	120	0.01	2857
H	60 µL of tube G	140	0.003	857
I	60 µL of tube H	120	0.001	285
J	60 µL of tube I	140	0.0003	85
K	60 µL of tube J	120	0.0001	28
L	60 µL of tube K	140	0.00003	8
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 is calculated. These represent the MAX signal in the assay. If LDL 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-Testosterone Acceptor Bead, SA-Donor Bead, and Buffer.

3. Preparation of 5X AlphaLISA Anti-Testosterone Acceptor beads (100 µg/mL):
    a. Add 10 µL of 5 mg/mL AlphaLISA Anti-Testosterone Acceptor beads to 490 µL of 1X AlphaLISA ImmunoAssay Buffer.
    b. Prepare just before use.

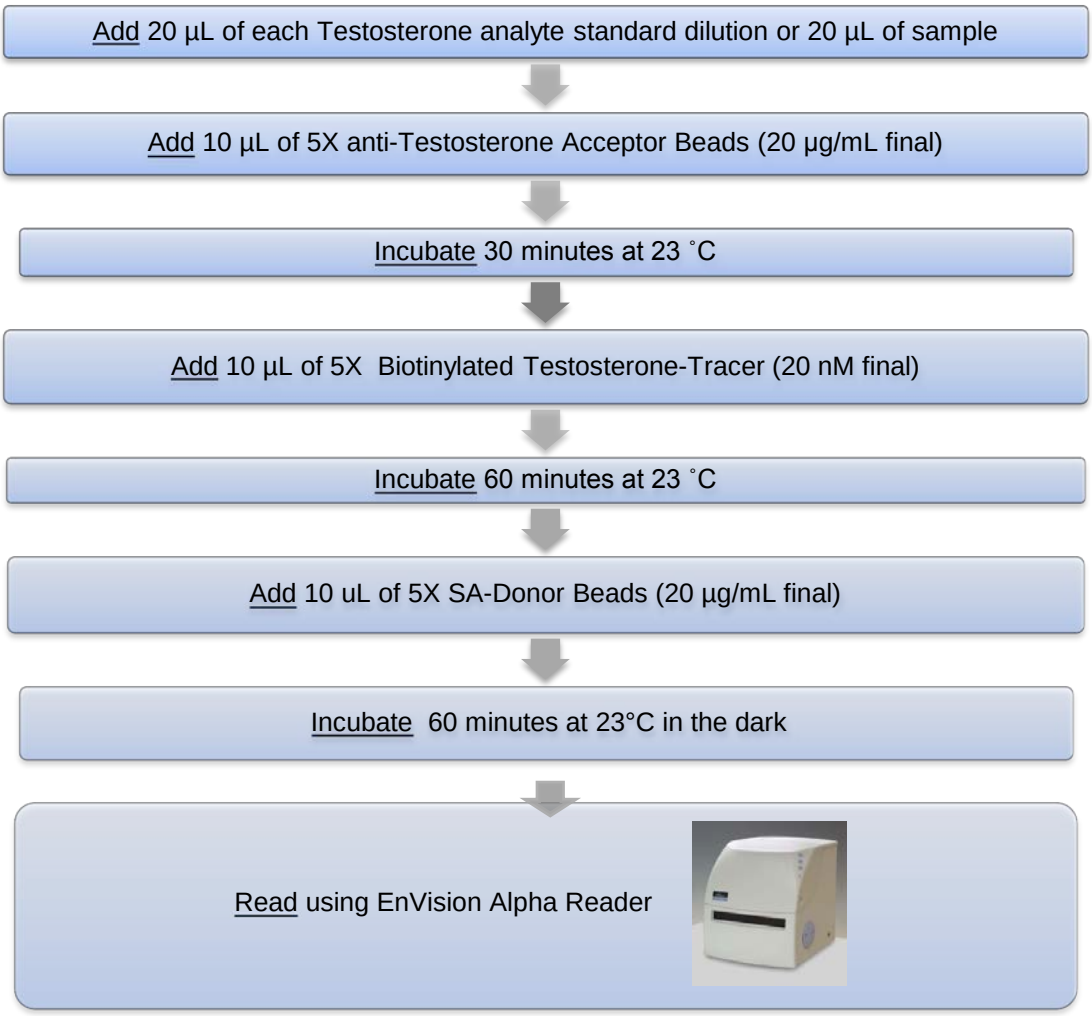
4. Preparation of 5X Biotinylated Testosterone-Tracer (100 nM):
    a. Add 1 µL of 100 µM Biotinylated Testosterone-Tracer to 999 µL of 1X AlphaLISA ImmunoAssay Buffer

5. Preparation of 5X Streptavidin (SA) Donor beads (100 µg/mL): Keep Donor beads under subdued laboratory lighting!
    a. Add 10 µL of 5 mg/mL SA-Donor beads 490 µL of 1X AlphaLISA ImmunoAssay Buffer
    b. Prepare just before use.

Samples:

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

In a 96- or 384-well microplate:



For High Sensitivity Protocol:

1) Preparation of 5X AlphaLISA Anti-Testosterone Acceptor beads (100 µg/mL):
    a. Add 10 µL of 5 mg/mL AlphaLISA Anti-Testosterone Acceptor beads to 490 µL of 1X AlphaLISA ImmunoAssay Buffer.
    b. Prepare just before use.

2) Preparation of 2.5X Streptavidin (SA) Donor beads (50 µg/mL) (**FOR NULL POINTS)**: Keep Donor beads under subdued laboratory lighting!
    c. Add 10 µL of 5 mg/mL SA-Donor beads 990 µL of 1X AlphaLISA ImmunoAssay Buffer
    d. Prepare just before use.

3) Preparation of 2.5X MIX [Streptavidin (SA) Donor beads (50 µg/mL) + Biotinylated Testosterone-Tracer (100 nM): Keep the beads under subdued laboratory lighting!

Add 10 µL of 5 mg/mL SA-Donor beads and 1.0 µL of 100 µM Biotinylated Testosterone-Tracer to 989 µL of 1X AlphaLISA ImmunoAssay Buffer

4) In a 96- or 384-well microplate: **(Hi sensitivity Protocol)**

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

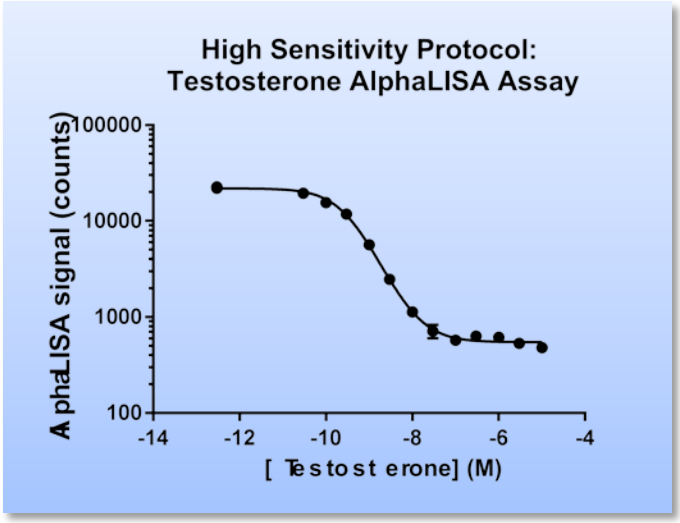
Add 10 µL of 5X anti-Testosterone Acceptor Beads (20 µg/mL final)

Incubate 60 minutes at 23 ˚C

Add 20 uL of 2.5X MIX :
SA-Donor Beads (20 µg/mL final) + Biotinylated Testosterone Tracer (40 nM final)

Incubate 60 minutes at 23°C in the dark

Read using EnVision Alpha Reader 

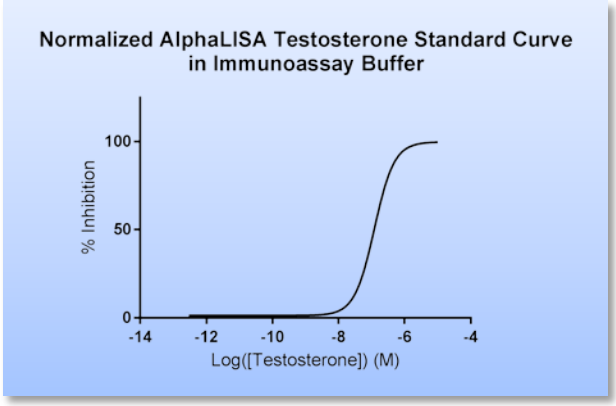


Data Analysis (Direct)

- 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.
- 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 Testosterone 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-Testosterone Acceptor Bead, Anti-Testosterone 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.
- 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 quick protocol.

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 20 µL using the recommended assay conditions.

LDL (nM)	Buffer/Media used	# of experiments
0.7	AlphaLISA ImmunoAssay Buffer	6
1.9	Charcoal stripped Serum	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 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 9 independent determinations in triplicate. Shown CV%.

Testosterone (nM)	IAB	Serum
30	3	12

- Inter-assay precision:

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

Testosterone (nM)	Buffer	Serum
30	8	14

- Spike Recovery:

Three known concentrations of analyte were spiked in ImmunoAssay Buffer, or human charcoal stripped serum. All samples, including non-spiked Immunoassay Buffers and charcoal stripped serum were measured in the assay. The average recovery from three independent measurements is reported.

Spiked Human IL-22 (nM)	% Recovery (in Immunoassay Buffer)	% Recovery (in CS Serum)
300	113 %	112 %
100	108 %	103 %
30	104 %	89 %

- Specificity:

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

Steroid	% Cross-reactivity
Prednisolone	0%
17-Hydroxyprogesterone	0%
Progesterone	0%
Cortisone	1%
Corticosterone	1%
21-deoxy-cortisol	0%
Cortisol	0%

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

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES

This product is not for resale or distribution except by authorized distributors.

PerkinElmer, Inc.
940 Winter Street
Waltham, MA 02451 USA
P: (800) 762-4000 or
(+1) 203-925-4602
www.perkinelmer.com