MANUAL

Technology: AlphaLISA®

AlphaLISA HEK 293 Host Cell Protein Detection Kit

Part number:	**AL3198HV**	**AL3198C**	**AL3198F**
Assay points:	100	500	5,000

Storage: Store kit in the dark at 4 °C. For reconstituted analyte, aliquot and store at -20 °C (possibility of 3 freeze-thaw cycles).

Version: 2 **Date:** January 2024

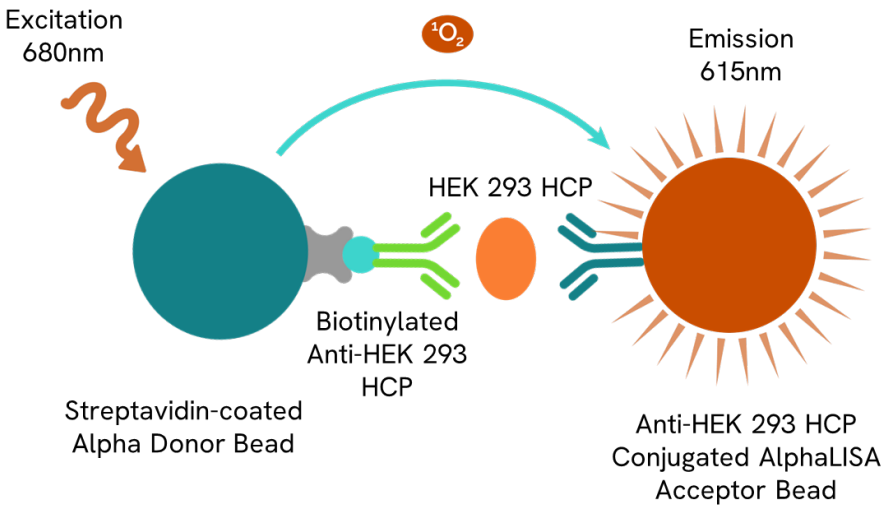
ANALYTE OF INTEREST

Host cell proteins (HCP) are process-related protein impurities found in drug product derived from host organisms (bacterial, yeast or mammalian production cell lines) during biotherapeutic manufacturing and purification. Among transgenic expression mammalian cell lines, Human Embryonic Kidney (HEK) 293 cells are commonly used. During products manufacturing by transgenic expression, HEK 293 cells can express many endogenous proteins, called HCP. Host cell protein contaminant clearance is a significant concern during downstream process development due to potential adverse clinical effects, decrease in product efficacy and stability. Hence, detection and quantification of HCP impurities is critical for biopharmaceutical companies in agreement with regulatory agency guidelines.

AlphaLISA HEK 293 Host Cell Protein Detection Kit is designed to quantitatively measure HCP contamination in products manufactured by transgenic expression in HEK 293 host cells.

DESCRIPTION OF THE ALPHALISA ASSAY

AlphaLISA technology allows the detection of molecules of interest in samples from manufacturing of product by transgenic expression in HEK 293 host cells, in a highly sensitive, quantitative, reproducible, and user-friendly mode. In this AlphaLISA assay, a biotinylated anti-HEK 293 HCP antibody binds to the streptavidin coated AlphaLISA Donor beads, while an anti-HEK 293 HCP antibody is conjugated to AlphaLISA Acceptor beads. In the presence of HEK 293 HCP, the beads come into proximity. The excitation of the Donor beads provokes the release of singlet oxygen molecules that triggers a cascade of energy transfer within the Acceptor beads, resulting in emission with λ_{max} at 615 nm.



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) can be applied to light fixtures.
- Take precautionary measures to avoid contamination of the reagent solutions.
- The biotinylated anti-HEK 293 HCP antibody contains sodium azide. Contact with skin or inhalation should be avoided.

KIT CONTENT: REAGENTS AND MATERIALS

Kit components	AL3198HV***	AL3198C***	AL3198F***
AlphaLISA anti-HEK 293 HCP Acceptor beads stored in PBS, 0.05% Kathon CG/ICP II, pH 7.2	30 µL @ 5 mg/mL (1 brown tube, white cap)	150 µL @ 5 mg/mL (1 brown tube, white cap)	1500 µL @ 5 mg/mL (1 brown tube, white cap)
Streptavidin (SA)-coated Donor beads stored in 25 mM HEPES, 100 mM NaCl, 0.05% Kathon CG/ICP II, pH 7.4	40 µL @ 5 mg/mL (1 brown tube, black cap)	200 µL @ 5 mg/mL (1 brown tube, black cap)	2 x 2 mL @ 5 mg/mL (2 brown tubes, black caps)
Biotinylated anti-HEK 293 HCP Antibody stored in PBS, 0.1% Tween-20, 0.05% NaN_3, pH 7.4	40 µL @ 500 nM (1 tube, black cap)	200 µL @ 500 nM (1 tube, black cap)	2000 µL @ 500 nM (1 tube, black cap)
Lyophilized HEK 293 HCP Standard*	1 µg (1 tube, clear cap)	1 µg (1 tube, clear cap)	1 µg (1 tube, clear cap)
AlphaLISA HiBlock Buffer (10X)**	2 mL, 1 small bottle	10 mL, 1 medium bottle	100 mL, 1 large bottle

* Reconstitute lyophilized analyte in 100 µL Milli-Q® grade H_2O **IMPORTANT: We recommend to vortex the analyte to homogenize solution.** The reconstituted analyte should be used within 60 minutes or aliquoted into screw-capped 0.5 mL polypropylene vials and stored at -20 °C for future experiments. The aliquoted analyte at -20°C is stable up to 4 weeks. It has been validated that three freeze-thaw cycles don't impact kit performance. One vial contains an amount of analyte sufficient for performing 10 standard curves. Additional vials of standard can be ordered separately (AlphaLISA HEK HCP Kit, std, cat # AL3198S).

** Extra buffer can be ordered separately (cat # AL004C: 10 mL, cat # AL004F: 100 mL).

*** The number of assay points is based on an assay volume of 50 µL 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 anti-HEK 293 HCP antibody stock solution will not interfere with the AlphaLISA signal (0.0001% final in the assay).

Additional reagents and materials:

The following materials are recommended but not provided in the kit:

Item	Suggested source
Light gray AlphaPlate™-384	Revvity Inc.
TopSeal™-A Plus Adhesive Sealing Film	Revvity Inc.
EnVision®-Alpha Reader	Revvity Inc.

RECOMMENDATONS

IMPORTANT: PLEASE READ THE RECOMMENDATIONS BELOW BEFORE USE

- 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 (2000*g*, 10-15 sec).
- Re-suspend the Donor and Acceptor beads by vortexing before use.
- Use Milli-Q® grade H_2O to dilute buffers and to reconstitute the lyophilized analyte. **IMPORTANT: We recommend to vortex the analyte to homogenize solution.**
- 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 Film to reduce evaporation during incubation. Microplates can be read with the TopSeal-A Film in place.
- The AlphaLISA signal is detected with an EnVision Multimode Plate 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. It is recommended to avoid multiple reads of the same well of the assay plate.
- The standard curves shown in this manual are provided for information only. A standard curve must be generated for each experiment.
- HEK 293 HCP standard (for standard curve) must be prepared in 1X AlphaLISA Hiblock Buffer or in the same medium as the samples. Some components used for biotherapeutics manufacturing may yield to slight interference in the assay. So, to reduce possible matrix effects, it is advised to dilute samples containing high HCP concentration in 1X AlphaLISA Hiblock Buffer. When testing samples without dilution, it is recommended to compare standard curves in both 1X AlphaLISA Hiblock Buffer

and sample's buffer. If the sample's buffer impairs standard curve results, we recommend running it in sample buffer instead of 1X AlphaLISA Hiblock Buffer.

ASSAY PROCEDURE

- 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 different amounts of samples are tested, the volumes of all reagents must 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.

Format	# of data points	Volume Final	Volumes for the 3-Step Protocol				Plate recommendation
			Sample	AlphaLISA Acceptor Beads	biotinylated Ab	SA-Donor beads	
AL3198HV	**100**	**50 µL**	**5 µL**	**10 µL**	**10 µL**	**25 µL**	**½ Area AlphaPlate-96**
AL3198C	250	100 µL	10 µL	20 µL	20 µL	50 µL	White OptiPlate-96
	500	**50 µL**	**5 µL**	**10 µL**	**10 µL**	**25 µL**	**½ Area AlphaPlate-96 White OptiPlate-384 Light gray AlphaPlate™-384**
	1 250	20 µL	2 µL	4 µL	4 µL	10 µL	Light gray AlphaPlate-384 ProxiPlate™-384 Plus White OptiPlate-384
AL3198F	**5 000**	**50 µL**	**5 µL**	**10 µL**	**10 µL**	**25 µL**	**½ Area AlphaPlate-96 White OptiPlate-384 Light gray AlphaPlate-384**
	12 500	20 µL	2 µL	4 µL	4 µL	10 µL	Light gray AlphaPlate-384 ProxiPlate-384 Plus White OptiPlate-384

The 3-Step protocol described below is for 500 assay points including one standard curve (48 wells) and samples (452 wells). *If different amounts of samples are tested, the volumes of all reagents must be adjusted accordingly.*

1) Preparation of 1X AlphaLISA Hiblock Buffer:
Add 5 mL of 10X AlphaLISA Hiblock Buffer to 45 mL Milli-Q® grade H_2O.

2) Preparation of HEK 293 HCP analyte standard dilutions:
   a. Reconstitute lyophilized [HEK 293 HCP, 1 µg] in 100 µL Milli-Q® grade H_2O. **IMPORTANT: We recommend to vortex the analyte to homogenize solution.** The remaining reconstituted

analyte should be aliquoted immediately and stored at -20 °C for future assays (see page 2 for more details).

b. A standard curve must be generated for each experiment. HEK 293 HCP standard (for standard curve) must be prepared in 1X AlphaLISA Hiblock Buffer or in the same medium as the samples. Some components used for biotherapeutics manufacturing may yield to slight interference in the assay. So, to reduce possible matrice effects, it is advised to dilute samples containing high HCP concentration in 1X AlphaLISA Hiblock Buffer. When testing samples without dilution, it is recommended to compare standard curves in both 1X AlphaLISA Hiblock Buffer and sample's buffer. If the sample's buffer impairs standard curve results, we recommend running it in sample buffer instead of 1X AlphaLISA Hiblock Buffer.

c. Prepare standard dilutions as follows (change tip between each standard dilution).

Tube	Vol. of HEK 293 HCP (µL)	Vol. of diluent (µL)*	[HEK 293 HCP] in standard curve	
			(g/mL in 5 µL)	(ng/mL in 5 µL)
A	10 µL of reconstituted HEK 293 HCP Standard	90	1 E-06	1 000
B	60 µL of tube A	140	3 E-07	300
C	60 µL of tube B	120	1 E-07	100
D	60 µL of tube C	140	3 E-08	30
E	60 µL of tube D	120	1 E-08	10
F	60 µL of tube E	140	3 E-09	3
G	60 µL of tube F	120	1 E-09	1
H	60 µL of tube G	140	3 E-10	0.3
I	60 µL of tube H	120	1 E-10	0.1
J	60 µL of tube I	140	3 E-11	0.03
K	60 µL of tube J	120	1 E-11	0.01
L	60 µL of tube K	140	3 E-12	0.003
M** (background)	0	50	0	0
N** (background)	0	50	0	0
O** (background)	0	50	0	0
P** (background)	0	50	0	0

* Dilute standards in appropriate diluent (e.g. 1X AlphaLISA Hiblock 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.

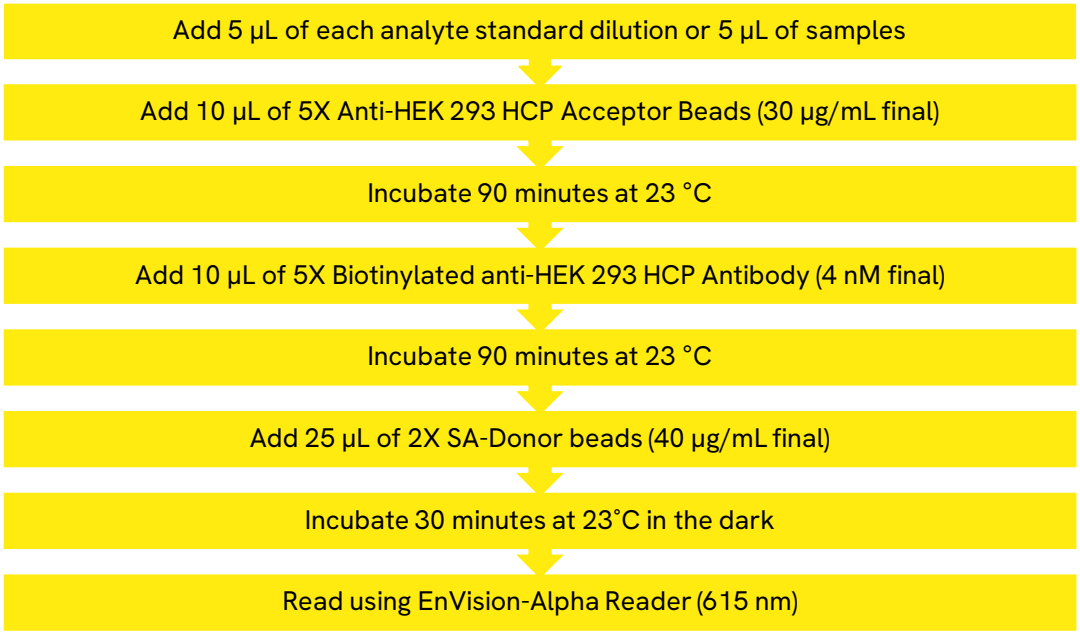
** Four background points in triplicate (12 wells) are used when LDL is calculated. If LDL does not need to be calculated, one background point in triplicate can be used (3 wells).

3) Preparation of 5X AlphaLISA Anti-HEK 293 HCP Acceptor beads (150 µg/mL)
    a. Prepare just before use.
    b. Add 150 µL of 5 mg/mL AlphaLISA Anti-HEK 293 HCP Acceptor Bead to 4 850 µL of 1X AlphaLISA Hiblock Buffer.

4) Preparation of 5X Biotinylated Anti-HEK 293 HCP Antibody (20 nM):
    a. Prepare just before use.
    b. Add 200 µL of 500 nM Biotinylated Anti-HEK 293 HCP Antibody to 4 800 µL of 1X

5) 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 12 300 µL of 1X AlphaLISA Hiblock Buffer.

6) In a light gray AlphaPlate (384 wells):



DATA ANALYSIS

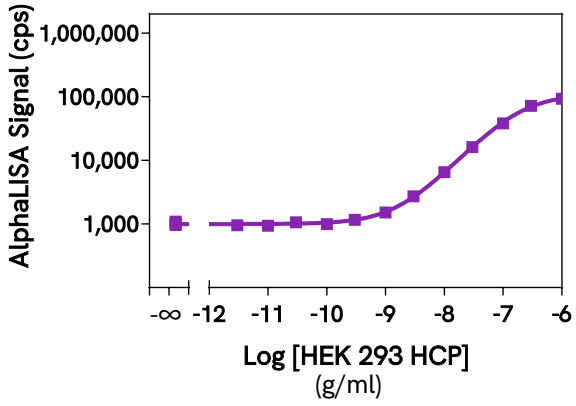
- Calculate the average count value for the background wells.
- Generate a standard curve by plotting the AlphaLISA counts versus the concentration of analyte. A log scale can be used for either or both axes. No additional data transformation is required.
- Analyze data according to a nonlinear regression 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 by using a 3-step protocol using 1X AlphaLISA Hiblock Buffer as assay buffer. All components, including standard curve and samples, were also prepared in 1X AlphaLISA Hiblock buffer. The assays were performed in 384-well plate format.

Standard curve:

A typical sensitivity curve is shown below, using the 3-step protocol described on page 6, using 1X AlphaLISA Hiblock Buffer to dilute the standard.



Assay sensitivity:

The LDL was 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 LDL corresponds to the lowest concentration of analyte that can be detected in a volume of 5 µL of sample using the recommended assay conditions.

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. The LLOQ corresponds to the lowest concentration level at which a measurement is quantitatively meaningful.

LDL (ng/mL)	*LLOQ (ng/mL)*
0.302	**1.013**

Assay precision:

Intra-assay precision:

The intra-assay precision was determined for each sample using a total of at least 22 independent determinations. Each replicate is interpolated in concentration on the standard curve and data of variability are shown as CV% of measured concentration.

Sample	*[HEK 293 HCP], ng/mL*	*CV%*
Sample A	11	**9%**
Sample B	23	**7%**
Sample C	46	**6%**
Sample D	93	**7%**
Sample E	183	**12%**
Sample F	359	**9%**
Sample G	611	**15%**

Inter-assay precision:

The inter-assay precision was determined for each sample using a total of 4 independent determinations with 3 replicates. Each replicate is interpolated in concentration on the standard curve and data of variability are shown as CV% as measured concentration.

Sample	*[HEK 293 HCP], ng/mL*	*CV%*
Sample A	10	**4%**
Sample B	21	**9%**
Sample C	43	**10%**
Sample D	87	**9%**
Sample E	159	**8%**
Sample F	344	**15%**

Antigen spike recovery:

Assay equilibrium between standard and sample was assessed using an antigen spike recovery experiment. Three different levels of HEK HCP standard were independently mixed with three different concentrations of a sample generated from a crude lysate from a biomanufacturing process using a subtype of HEK 293 cell line. All assay components, including standard curve, were diluted in 1X AlphaLISA Hiblock buffer. Concentrations of HEK 293 HCP in samples (= measured concentrations) were determined by interpolating concentrations to the standard curve. Total measured concentrations were compared to the theoretical ones (= expected concentration) and expressed as % antigen recovery. Excellent antigen spike recovery was achieved in all tested conditions (acceptance criteria: 85-115%). The results are shown in the table below.

[HEK 293 HCP standard], ng/mL	*[HEK 293 HCP sample], ng/mL*	*Expected [HEK 293 HCP], ng/mL*	*Measured [HEK 293 HCP], ng/mL*	*% antigen recovery*
166.7	219.4	386.1	408.3	**106%**
	125.8	292.5	246.0	**84%**
	69.1	235.8	210.9	**89%**
40.6	69.1	109.6	103.6	**95%**
	35.5	76.0	76.3	**100%**
	17.7	58.3	56.2	**96%**
10.2	17.7	27.9	26.7	**96%**
	8.1	18.3	19.4	**106%**
	5.0	15.2	13.3	**88%**

Dilution Linearity:

Two samples corresponding to crude lysates from 2 HEK 293 subtypes were prepared with 1X AlphaLISA Hiblock buffer and serial dilutions of the samples were made with a 2- to 256-fold dilution using 1X AlphaLISA Hiblock buffer. All assay components, including standard curve, were prepared in 1X AlphaLISA Hiblock buffer. Concentrations of HEK 293 HCP in diluted samples were determined by interpolating concentrations to the standard curve. Excellent dilution linearity was achieved in the two samples (2- to 64 or 256-fold dilution) with a global mean % dilution recovery closed to 100%. The results are shown in the tables below.

Sample A			
Dilution Factor (x)	**Expected [*HEK 293 HCP*], ng/mL**	**Measured [*HEK 293 HCP*], ng/mL**	**% dilution recovery**
Neat	193.1	193.1	**100%**
2	96.6	97.6	**101%**
4	48.3	50.1	**104%**
8	24.1	25.3	**105%**
16	12.1	12.5	**104%**
32	6.0	6.1	**101%**
64	3.0	3.1	**103%**
Linearity R^2 = 0.9999			

Sample B			
Dilution Factor (x)	**Expected [*HEK 293 HCP*], ng/mL**	**Measured [*HEK 293 HCP*], ng/mL**	**% dilution recovery**
Neat	609.9	609.9	**100%**
2	305.0	285.6	**94%**
4	152.5	148.2	**97%**
8	76.2	82.3	**108%**
16	38.1	42.6	**112%**
32	19.1	18.7	**98%**
64	9.5	10.3	**108%**
128	4.8	4.2	**88%**
256	2.4	2.6	**108%**
Linearity R^2 = 0.9988			

TROUBLESHOOTING

To find detailed recommendations for common situations you might encounter with your AlphaLISA assay kit, please visit our website at www.revvity.com.

www.revvity.com

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