APPLICATION NOTE

Streamline the HEK 293 HCP impurity quantification workflow with new, no-wash AlphaLISA HEK 293 Host Cell Protein Kit

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Summary

Host cell proteins (HCP) are process-related protein impurities present in drug products derived from host organisms, including bacterial, yeast, or mammalian production cell lines. This occurs during the manufacturing and purification of Adeno-Associated Virus (AAV) or recombinant proteins. Human Embryonic Kidney (HEK) 293 cells are commonly used in the expression and production of therapeutic proteins and viruses for gene therapy. During the transgenic expression manufacturing process, HEK 293 cells have the capability to express numerous endogenous proteins, referred to as HCPs. The clearance of HCP contaminants is a significant concern during downstream process development due to potential adverse clinical effects, a decrease in product efficacy, and stability concerns. Therefore, the detection and quantification of HCP impurities are critical for biopharmaceutical companies in agreement with regulatory agency guidelines.

Addressing this concern, the AlphaLISA™ HEK 293 Host Cell Protein Detection Kit has been designed to quantitatively measure HCP contamination in products manufactured by transgenic expression in HEK 293 host cells. The assessment covers the entire spectrum, from crude harvest material to the final product, offering a quantitative, reproducible, and user-friendly mode (Table 1).

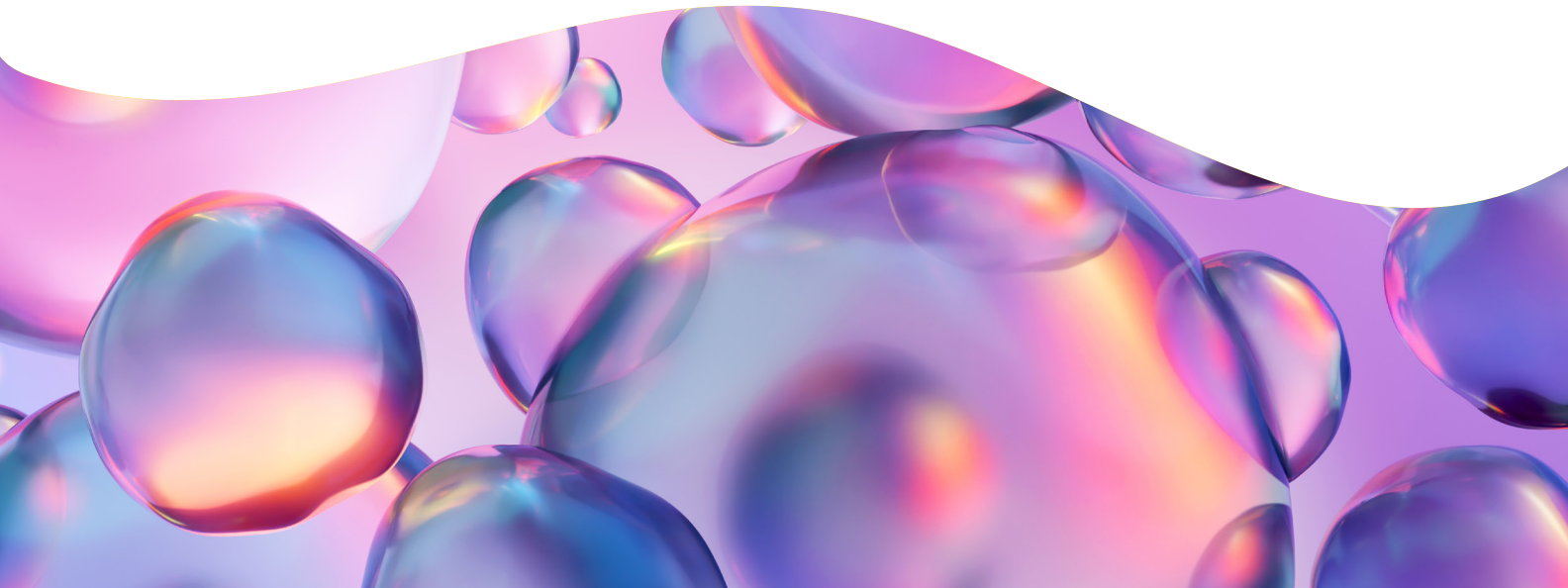

Table 1: Specifications of the AlphaLISA HEK 293 HCP detection kit

	AlphaLISA - AL3198HV/C/F
Format	Homogeneous assay
LOD (ng/mL)	0.302 ng/mL*
LOQ (ng/mL)	1.013 ng/mL**
Standard range (ng/mL)	0.003-1,000 ng/mL
Quantitative range (ng/mL)	1.013-1,000 ng/mL
Time to results	3h30

* The LOD 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 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 LOQ 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.

Principle of the AlphaLISA HEK HCP 293 Host Cell Protein Detection Kit

In the AlphaLISA assay, the anti-HEK 293 HCP polyclonal antibody is biotinylated on one side to bind with streptavidin coated AlphaLISA Donor beads and conjugated to AlphaLISA Acceptor beads on the other side. In the presence of HEK 293 HCPs, the beads come into proximity. The excitation of the Donor beads provokes a release of singlet oxygen molecules that triggers a cascade of energy transfer within the Acceptor beads, resulting in emission with a peak wavelength (λmax) at 615 nm (Figure 1).

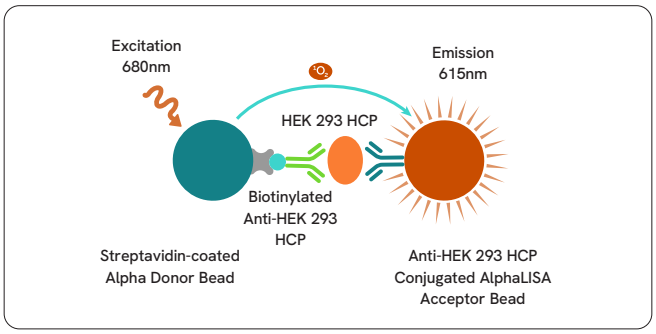

Figure 1: AlphaLISA HEK 293 HCP detection kit principle

Good dilutional linearity and antigen spike recovery

Dilutional linearity and antigen spike recovery experiments are important methods for assessing and verifying the performance of immunoassays.

Dilutional linearity

Dilutional linearity experiments are performed to demonstrate the capability of the immunoassay and its optimized diluent to effectively dilute highly concentrated samples down to the standard curve, while still giving reliable expected results when multiplied by the dilution factor. This is particularly important when immunoassays are intended to measure complex analytes. Considering the diverse nature of HCPs and the potential presence of very high concentrations of HCPs (beyond the maximum quantitation assay range), dilutional linearity is an important factor to be verified.

To assess the dilutional linearity of this AlphaLISA HEK 293 HCP detection kit, two samples corresponding to two HEK 293 subtypes were prepared with the appropriate assay buffer (1X AlphaLISA Hiblock buffer). Subsequently, serial dilutions of these samples were made with the assay buffer. The concentrations of HEK 293 HCPs in diluted samples were determined by interpolating concentrations with the standard curve. Excellent dilution linearity was achieved in the two samples (2- to 64-fold or 256-fold dilution) with a global mean % dilution recovery close to 100%. Additionally, the coefficient of the linear regression between measured and expected concentrations was evaluated. The results obtained are shown in the tables below (Table 2).

Table 2: Dilutional recovery (%) for two samples from two HEK 293 subtypes and diluted as indicated with1X AlphaLISA Hiblock buffer

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			

Antigen spike recovery

An antigen spike recovery test is another metric of immunoassay performance used to investigate whether substances are present in the sample that could interfere with the measurement and to assess the assay's ability to recover added/spiked analytes. This test also verifies the consistency of responses and assay equilibrium between standards and biological samples.

In this study, three distinct levels of HEK 293 HCP standard were independently mixed with three concentrations of a sample derived from a crude lysate from a biomanufacturing process using a specific subtype of the HEK 293 cell line. The concentrations of HEK 293 HCPs in the samples (= measured concentrations) were determined by interpolating concentrations with the standard curve. The total measured concentrations were compared to theoretical values (= expected concentration) and expressed as % antigen recovery. The analysis demonstrated excellent antigen spike recovery under all tested conditions (acceptance criteria: 85-115%) (Table 3).

Table 3: Antigen recovery (%) for a mixture of HEK 293 HCP standard with a sample generated from a crude lysate from a biomanufacturing process using a subtype of HEK 293 cell line

[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%**

Precision

As shown in Table 4 and Table 5, the HEK 293 HCP detection kit is a robust assay with reproducible results (%CV intra and %CV inter on concentrations < 15%).

Intra-assay precision (reproducibility)

The intra-assay precision was determined for each sample using at least 22 replicates. Each replicate was interpolated in concentration on the standard curve and the variability data are presented as a percentage of the coefficient of variation (CV%) of the measured concentrations (Table 4).

Table 4: Intra-assay precision of AlphaLISA HEK 293 HCP detection kit

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

Inter-assay precision (repeatability)

The inter-assay precision was determined for each sample using a total of four independent experiments with three replicates per experiment. Each replicate was interpolated in concentration on the standard curve and variability data are presented as a CV% of the measured concentrations (Table 5).

Table 5: Inter-assay precision of AlphaLISA HEK 293 HCP detection kit

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

Compatibility with buffers commonly used in AAV manufacturing

Some components used in AAV or recombinant protein manufacturing buffers (low pH, high salt concentration, excipients, etc.) may slightly interfere with the immunoassay. Consequently, we recommend evaluating the potential matrix effect associated with each buffer tested, particularly during initial usage. To assess potential interferences, we advise comparing the standard curves in the assay buffer and the investigated manufacturing buffer. The concentrations of the standard curve prepared in the investigated buffer are compared with the expected concentrations (= concentration of the standard curve in the assay buffer) and expressed as % antigen recovery. We consider that an acceptable antigen recovery should fall between 80% and 120%.

Case 1: Matrix effect is highlighted, and sample contains elevated HCP concentrations

In instances where a matrix effect is highlighted and the sample contains a high concentration of HCPs, we advise diluting the sample in the assay buffer to reduce possible matrix effects. The optimal dilution factor can be determined by preparing a HEK 293 HCP sample spiked in the buffer of interest, followed by a series of sample dilutions in the assay buffer. Concentrations of HEK 293 HCPs in these samples (= measured concentrations) can be determined by interpolating concentrations with the standard curve (HEK 293 HCP standard diluted in the assay buffer. Subsequently, the measured concentrations are compared to the expected values (= concentration of HEK 293 HCP sample spiked in the assay buffer) and expressed as % antigen recovery. We consider the dilution factor to be optimal when antigen recovery falls between 80% and 120%. Table 6 below shows examples of data for several commonly used AAV manufacturing buffers assessed with the AlphaLISA HEK 293 HCP detection kit. For each AAV manufacturing buffer investigated, the selected dilution factor is highlighted.

After screening the optimal dilution factor for the AAV manufacturing buffer of interest, it is recommended to further confirm this dilution factor. This can be achieved by spiking various concentrations of HEK 293 HCPs into the buffer under investigation, then diluting them by the determined dilution factor with the assay buffer. The next step is to calculate the antigen recovery (acceptance criteria: 80-120%). Table 7 below shows examples of data for several commonly used AAV manufacturing buffers assessed with the AlphaLISA HEK 293 HCP detection kit.

Table 6: Antigen recovery (%) of HEK 293 HCP sample spiked in several buffers commonly used in AAV manufacturing and diluted in 1X AlphaLISA Hiblock buffer as indicated

AAV manufacturing step	Buffer composition	Dilution factor	Expected [HEK 293 HCP], ng/mL	Measured [HEK 293 HCP], ng/mL	Antigen recovery
Harvest	50 mM Tris-HCl, pH 8.5, 150 mM NaCl, 5 mM MgCl2, 0.001% Pluronic F-68, 0.1% Triton X-100, 25 units/mL Benzonase	**Neat**	**356**	**305**	**86%**
Capture chromatography	1/2 (100 mM Citrate pH 2.0) + 1/2 (0.45M Tris pH 8.0 + 150 mM NaCl), pHf 6.2	Neat	879	58	7%
		2	439	117	27%
		50 (Neat = 2000)	**439**	**356**	**81%**
Polishing	40% Iodixanol, 0.33X PBS-MK, 0.001% Pluronic F-68	Neat	279	16	6%
		2	158	30	19%
		50 (Neat = 2000)	**390**	**349**	**89%**
Formulation	PBS, 0.014 % Pluronic F-68	Neat	356	223	62%
		2	**178**	**160**	**90%**

Table 7: Antigen recovery (%) of HEK 293 HCP sample spiked in several buffers commonly used in AAV manufacturing, then diluted in 1X AlphaLISA Hiblock buffer as indicated

AAV manufacturing step	Buffer composition	Dilution Factor	Expected [HEK 293 HCP], ng/mL	Measured [HEK 293 HCP], ng/mL	Antigen recovery
Harvest	50 mM Tris-HCl, pH 8.5, 150 mM NaCl, 5 mM MgCl2, 0.001% Pluronic F-68, 0.1% Triton X-100, 25 units/mL Benzonase	Neat	114	120	**105%**
		Neat	235	238	**101%**
		Neat	400	414	**104%**
Capture chromatography	1/2 (100 mM Citrate pH 2.0) + 1/2 (0.45M Tris pH 8.0 + 150 mM NaCl), pHf 6.2	50	114	98	**86%**
		50	235	190	**81%**
Polishing	40% Iodixanol, 0.33X PBS-MK, 0.001% Pluronic F-68	50	114	123	**108%**
		50	235	262	**112%**
		50	400	470	**118%**
Formulation	PBS, 0.014% Pluronic F-68	2	114	111	**97%**
		2	235	209	**89%**
		2	400	394	**99%**

Case 2: Matrix effect is highlighted and the sample cannot be diluted due to an HCP concentration close to the assay's lowest limit of detection

If a matrix effect is found and the sample cannot be diluted as the HCP content is close to the assay's limit of detection, we recommend running the standard curve in the same buffer as that used in the sample.

Lack of cross-reactivity between HEK 293 HCP detection and AAV presence

The AlphaLISA HEK 293 HCP detection kit was checked for its putative cross-reactivity with AAVs. Two AAV subptypes commonly used in clinical applications (AAV2 and AAV5) were spiked into HEK 293 HCP samples with different concentrations of HCPs. As reported in Table 8, no significant impact on antigen recovery (≤ 20%) was observed.

Table 8: Antigen recovery (%) for HEK 293 HCP samples in presence of two AAV subtypes (AAV2 and AAV5)

	Antigen recovery	
[HEK 293 HCP], ng/mL	5 X10^{11} /mL AAV2	5 X10^{12} /mL AAV5
75	109%	116%
150	109%	118%
300	91%	117%

Antibody coverage analysis

An important analysis to verify HCP detection is the determination of antibody coverage, offering a means to estimate the percentage of total HCPs that the assay can detect. Although regulatory authorities do not provide guidance concerning a minimum coverage percentage, it is beneficial to demonstrate that the assay's polyclonal antibody pool displays the broadest coverage percentage. For a generic kit, achieving a 60% coverage is considered to be sufficient, though it is recommended to obtain the highest coverage percentage for your manufacturing workflow.

In our investigation, we performed a coverage analysis by 2D-DIBE (2D Differential in Blot Electrophoresis) on two crude lysates derived from a biomanufacturing process using two subtypes of the HEK 293 cell line with the Revvity anti-HEK 293 HCP polyclonal antibody. Proteins from the two HEK 293 subtypes were independently labeled with Cy3. The labeled protein samples were then separated based on their isoelectric point and molecular weight using the 2-dimensional SDS-PAGE technique. 2D gels were then transferred to a Western Blot membrane and incubated with the Revvity anti-HEK 293 HCP polyclonal antibody. Detection of bound antibodies was achieved using Cy5-labeled secondary antibodies raised against the primary antibody's host species. The membranes were scanned on an imager and images were acquired for both Cy3 and Cy5 channels. Spot detection and antibody coverage were assessed using dedicated software. As shown in Figure 2 and Table 9, the Revvity anti-HEK 293 HCP polyclonal antibody has an excellent coverage of HEK 293 HCPs across the two HEK 293 subtypes tested.

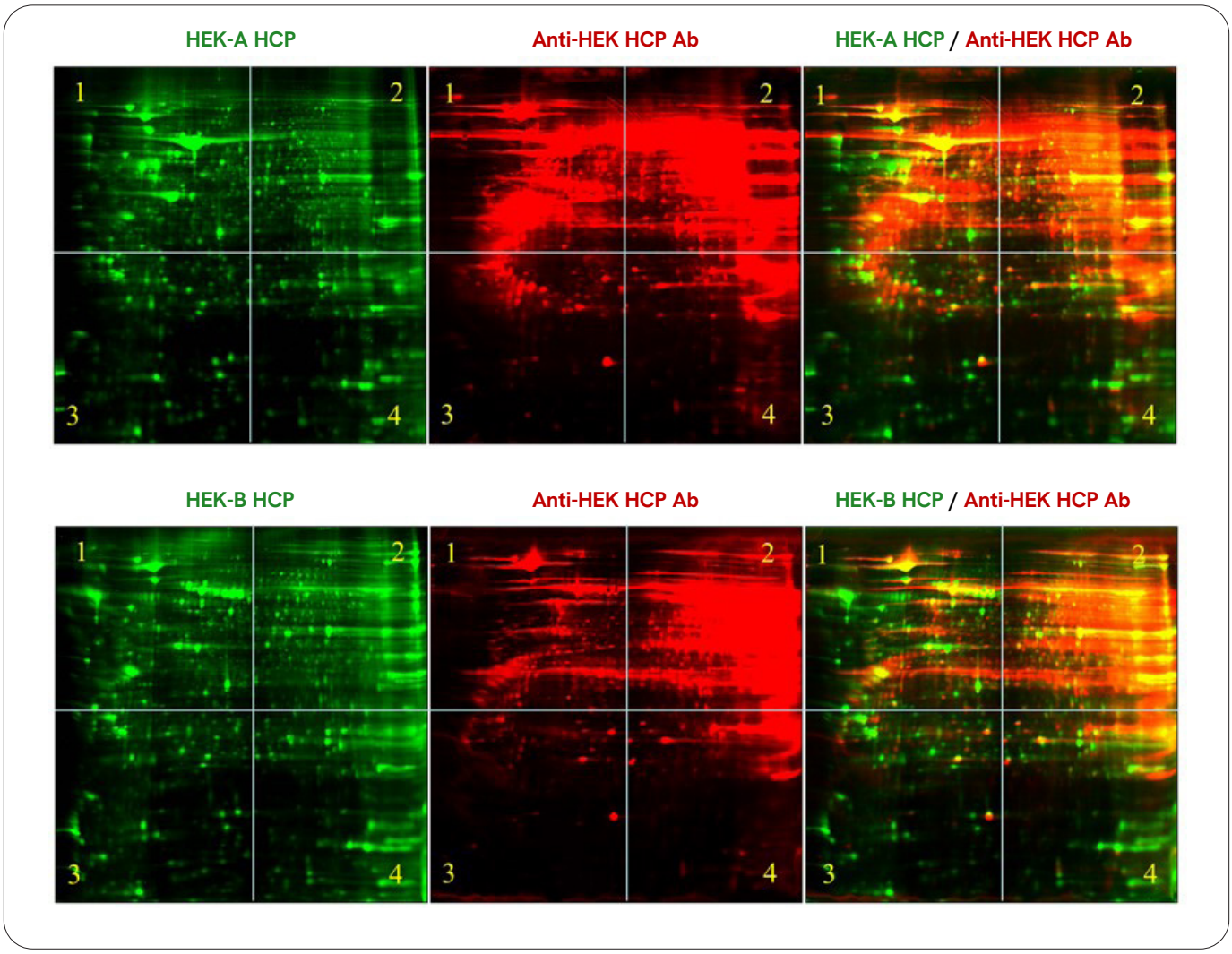

Figure 2 : Coverage of HEK 293 HCPs derived from two subtypes of HEK 293 cell lines commonly used in AAV or recombinant protein manufacturing with the Revvity anti-HEK 293 HCP polyclonal antibody

Table 9: % coverage of HEK 293 HCPs derived from two subtypes of HEK 293 cell lines commonly used in AAV or recombinant protein manufacturing with the Revvity anti-HEK 293 HCP polyclonal antibody

	HEK 293 subtypes	
	A	B
% total coverage of HEK 293 HCPs by 2D-DIBE	98.7%	96.0%

Conclusion

This application note demonstrates the ability of the ready-to-use AlphaLISA no-wash HCP detection assay kit to quantify HEK 293 HCP impurities quickly and easily during biopharmaceutics manufacturing. This off-the-shelf kit is designed to deliver a streamlined workflow and a broader dynamic range compared to traditional multi-step ELISA assays.

The assay displays exceptional dilutional linearity, reliable antigen spike recovery, and robust reproducibility. The kit is compatible with the most commonly used biotherapeutics manufacturing buffers and in presence of the drug substance.

The excellent coverage of HEK 293 HCPs demonstrated here underscores the efficacy of this innovative AlphaLISA assay kit, enhancing existing workflows and contributing to quality control measures and the overall success of biotherapeutic production.

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